

Chemical and Proteolytic Modifications of Mitochondrial Aspartate Aminotransferase

**Identification of Arg 292 as the Binding Site
for the Distal Carboxylate Group of the Substrate**

**Aspartate Aminotransferase 27/32 – 410, a Partially Active
Enzyme Derivative Produced by Limited Proteolysis**

INAUGURAL-DISSERTATION
zur Erlangung der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der
Universität Zürich

von
ERIKA SANDMEIER
aus Seengen AG

Begutachtet von Herrn Prof. Dr. Ph. Christen



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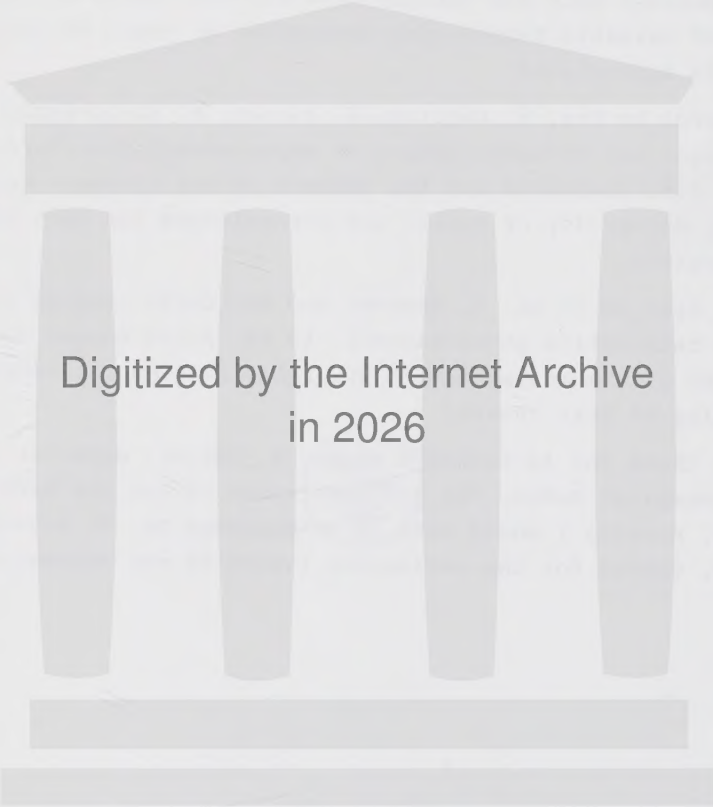
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Parts of this work have already been published elsewhere:

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Mitochondrial Aspartate Aminotransferase 27/32 - 410: Partially Active Enzyme Derivative Produced by Limited Proteolytic Cleavage of Native Enzyme. Sandmeier, E., and Christen, P. (1980) J. Biol. Chem. 255, 10284-10289.

Functional Groups at the Active Site of Mitochondrial Aspartate Aminotransferase (mAATase). Eichele, G., Ford, G.C., Jansonius, J.N., Sandmeier, E., Hausner, U., Wilson, K.J., and Christen, P. 12. Jahresversammlung der USGEB, Basel, 1980, Abstract, *Experientia* 36, 720 (1980) and 13th Meeting of the Federation of European Biochemical Societies, Jerusalem, 1980, Abstracts, p. 138.

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Chemical Modification of a Functional Arginyl Residue (Arg 292) of Mitochondrial Aspartate Aminotransferase. Identification as the Binding Site for the Distal Carboxylate Group of the Substrate. Sandmeier, E., and Christen, P., submitted for publication.

Abbreviations

AspAT Aspartate aminotransferase

mAspAT Aspartate aminotransferase
 (mitochondrial isoenzyme)

PLP Pyridoxal-5'-phosphate

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INTRODUCTION

Aspartate aminotransferase (AspAT) is a prototype of the substantial number of pyridoxal-5'-phosphate (vitamin B₆)-dependent enzymes which catalyze manifold reactions in the metabolism of amino acids. It is also the first representative of this group of enzymes for which a detailed model of its three-dimensional structure has been obtained by high-resolution x-ray crystallographic analysis. Conjointly, investigations of the enzyme in solution and x-ray crystallographic studies have provided an experimental framework within which current efforts are aimed to describe in detail the mechanism of action of this enzyme.

The subject of this study is the exploration of the rôle of functional residues at the active site and of certain residues not situated at the active site for the catalytic function of the mitochondrial aspartate aminotransferase (mAspAT). Both chemical and proteolytic modifications were used to define such residues or regions. Chemical modification with phenylglyoxal revealed that a particular arginyl residue which was identified as Arg 292 functions in the binding of the distal carboxylate group of the substrate. The proteolytic modification of mAspAT indicated that the NH₂-terminal segment is of both structural and functional significance. In addition to stabilizing the quaternary structure this part of the polypeptide chain appears to have a rôle in propagating syncatalytic conformational rearrangements.

1. Aspartate Aminotransferase as Prototype of a Pyridoxal-5'-Phosphate-Dependent Enzyme

General Mechanism of Pyridoxal-5'-Phosphate-Dependent Enzymes

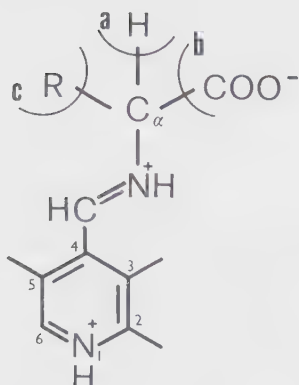
Pyridoxal-5'-phosphate (PLP), the biochemically functional derivative of vitamin B₆, is the prosthetic group of a large group of enzymes which synthesize, degrade and interconvert amino acids. In all of these reactions, PLP functions according to the same general mechanism as proposed by Braunstein and Shemyakin (1) and by Metzler, Ikawa and Snell (2). Their description of the mechanism is based on studies of nonenzymic reactions of amino acids with free pyridoxal. Its first step is the reaction of the aldehyde group of pyridoxal with the amino acid to form a Schiff base. The heterocyclic nitrogen serves as a strong electron-attracting group which enables the displacement of an electron pair from the α -carbon atom of the amino acid toward the pyridine ring.

The formation of a Schiff base is common to all PLP-dependent enzymes. In these enzymes, however, PLP is not present as free aldehyde but is bound via a Schiff base linkage to the ϵ -amino group of a lysyl residue (for a review see Ref. 3). The formation of the Schiff base with the substrate thus corresponds to a transaldimination.

PLP-dependent enzyme reactions can be classified in three main groups with regard to the bond of the substrate (a, b or c in Scheme 1) which is cleaved after forming the Schiff base. About 10 to 12 different reaction types are known (4).

The apoenzyme determines the type of the reaction which is realized. A hypothetical relationship between the reaction specificity and structural features of the active site was proposed by Dunathan (5). The bond which is broken in the

SCHEME I



- a) Deprotonation at the α -carbon atom. The ensuing quinonoid intermediate may undergo one of the following reactions:
transamination (over 50 transaminases are known)
racemization (racemases)
 β -elimination (e.g. serine dehydratase, threonine dehydratase)
 β -replacement (e.g. tryptophan synthase, cysteine synthase)
- b) Decarboxylation, i.e., breaking of the C_{α} -COOH bond (amino acid decarboxylases)
- c) Side chain cleavage, i.e., aldol cleavage (e.g. serine transhydroxymethylase)

coenzyme-substrate intermediate must be orientated perpendicular to the plane of the pyridine ring. In this conformation the breaking bond achieves maximal sigma-pi overlap with the ring-imine pi-system. In transaminases, according to this postulate, the C_{α} -H $_{\alpha}$ bond will lie orthogonal to the conjugated pi-system.

Hypothetical Mechanism of Aminotransferases

The main steps in the mechanism of transamination between 2-oxo acids and amino acids can be summarized as follows (Scheme II, for a review see Ref. 6):

Transaldimination

- PLP, covalently bound to the ϵ -amino group of Lys 258 by a Schiff base linkage (internal aldimine, I).
- Nucleophilic addition of the substrate to the $>C=N$ bond. Formation of a tetrahedral intermediate (II).
- Release of Lys 258 yielding the external aldimine (III).

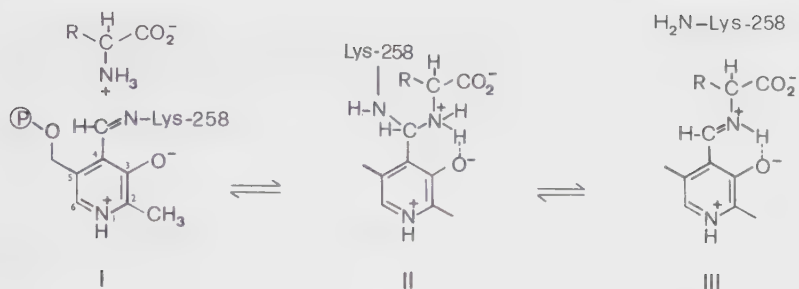
Tautomerization

- Elimination of the proton at the α -C from the external aldimine (III) yields a semiquinonoid intermediate (IV) which includes three resonance states (viz. the α -C-carbanion of the aldimine, a planar semiquinonoid structure and the 4'-C-carbanion of the ketimine).
- Reprotonation at the 4'-C-carbon results in formation of a ketimine (V).

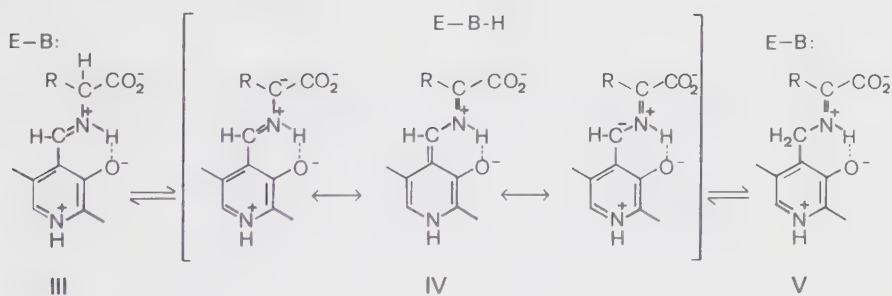
The tautomerization, the rate-limiting step of the enzymic transamination reaction, is base-catalyzed.

SCHEME II

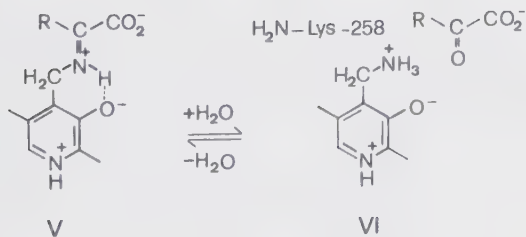
Transaldimination



Tautomerization



Hydrolysis



Hydrolysis

- The $C_{\alpha}=N$ double bond of the ketimine (V) is hydrolyzed yielding the pyridoxamine form (VI) of the enzyme and the 2-oxo acid product.

In the second half-reaction of transamination the pyridoxamine form (VI) reacts with another 2-oxo acid regenerating the pyridoxal form by the same steps in reverse succession and forming a new amino acid product.

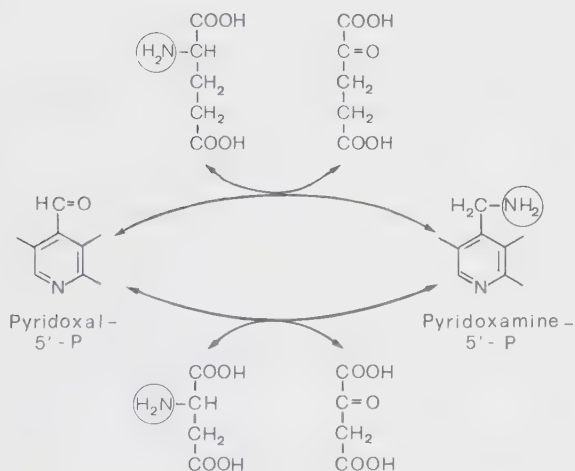
This mechanism has been verified by numerous experimental data obtained from nonenzymic model reactions as well as from studies of enzymic transamination.

Enzymic transamination is accompanied by conformational rearrangements of the enzyme-coenzyme-substrate complex. Ivanov and Karpeisky (7) were the first to suggest a rotatory displacement of the coenzyme. Such a movement of the pyridine ring during catalysis was confirmed by x-ray crystallographic studies at 2.8 Å (8) and by measurement of the linear dichroism of the coenzyme chromophore with single crystals (9, 10). Conformational changes of the protein moiety during catalysis were observed in experiments with the enzyme in solution. Both Cys 390 in the cytosolic and Cys 166 in the mitochondrial isoenzyme show alterations in their reactivity toward modifying reagents in the different enzyme-substrate intermediates (11, 12). A third syncatalytic conformational adaption of the enzyme has been found by infrared spectrophotometric measurement of peptide hydrogen-deuterium exchange (13). The relationship of these changes in conformation to the motion of the so-called small domain observed in crystallographic studies (8) is currently under investigation.

Catalytic and Structural Properties of Aspartate Aminotransferase

AspAT is the most extensively studied transaminase. The enzyme converts L-glutamate or L-aspartate to 2-oxoglutarate or oxalacetate, respectively, by a double displacement reaction (Scheme III) during which the coenzyme shuttles between the pyridoxal form and the pyridoxamine form according to the mechanism described above.

SCHEME III



In animal tissues two homologous isoenzymes of AspAT have been found, one located in the cytosol, the other in the mitochondria. The mitochondrial enzyme is synthesized on cytosolic ribosomes as a precursor with higher molecular weight (14). The mechanism of its translocation from the site of synthesis into mitochondria is under investigation (15, 16).

Since 1959, when AspAT was isolated for the first time in a highly purified state as the first PLP-dependent enzyme (17), numerous studies on the structural and chemical topography of the active site, the conformational rearrangements and the catalytic mechanism have been published (for a review see Braunschtein, Ref. 6). An important step was the determination of the complete amino acid sequence of AspAT. In the last eight years the following six primary structures of AspAT have been published:

TABLE I Known amino acid sequences of aspartate aminotransferases

Isoenzyme	Source	References
Cytosolic	Pig	Ovchinnikov et al., 1973 (18)
Cytosolic	Pig	Doonan et al., 1975 (19)
Cytosolic	Chicken	Shlyapnikov et al., 1979 (20)
Mitochondrial	Turkey	Barra et al., 1979 (21) (determined to 75%)
Mitochondrial	Pig	Kagamiyama et al., 1980 (22)
Mitochondrial	Rat	Huynh et al., 1980 (23)
Mitochondrial	Chicken	Graf-Hausner et al. (24)

Comparison of these amino acid sequences indicates that the mitochondrial and the cytosolic isoenzymes are homologous proteins, i.e., have arisen from a common ancestor protein. In the last few years many PLP-dependent enzymes have been crystallized (for a review see Yang and Metzler, Ref. 25), but so far only three aspartate aminotransferases have been studied by high resolution x-ray crystallographic analysis, namely the cytosolic enzyme from chicken (26) and from pig (27) and the mitochondrial isoenzyme from chicken (8). Sum-

marizing the x-ray crystallographic data, mAspAT from chicken can be characterized as follows: The enzyme is an α_2 dimer with 2 x 401 amino acid residues (see Footnote). Each subunit consists of two domains, the larger one containing the coenzyme PLP. The two active sites are situated on opposite sides of the enzyme dimer in clefts near the subunit interface. Each active site appears to be composed of structural elements of both subunits (see Fig. 13 in the Discussion section). The amino acid residues which were found to be in or near the active site (8) are conserved in all six known sequences with the exception of Trp 140 which is a glycyl residue in the mitochondrial enzyme from rat (23) and Ser 107 which in both cytosolic enzymes is substituted by a glycyl residue (18-20).

2. Chemical Modifications of Aspartate Aminotransferase

The chemical modification of amino acid residues is an informative method for characterizing functional groups of proteins. In AspAT the first and only amino acid residue to be located at the active site by means of chemical modification was the lysyl residue whose ϵ -amino group forms the Schiff base linkage to PLP (28-30). On the basis of the first determined amino acid sequence (18) this functional residue was identified as Lys 258.

Footnote: The numbering of amino acid residues of mAspAT corresponds to that of the cytosolic isoenzyme from pig which has a total of 412 residues per polypeptide chain (18, 19). Due to several deletions, there are only 401 residues in mAspAT which are numbered from 3 to 410 (Ref. 24 for the mAspAT from chicken and Ref. 22 for the mitochondrial isoenzyme from pig).

Chemical modification with tetranitromethane preferentially affected the apoenzyme (31, 32). Because tetranitromethane not only modifies tyrosyl residues, but also cysteinyl, tryptophanyl and methionyl residues (33, 34), the interpretation of the reaction with this reagent seems ambiguous. Apparently, the residue nitrated in the holoenzyme which was identified as Tyr 40 (35, 36) is a secondary event facilitated by oxidation of Cys 390 (37). By using the crosslinking reagent 1,5-difluoro-2,4-dinitrobenzene, another tyrosyl residue, possibly Tyr 70, was found to lie at the active site close to Lys 258 (38), a conclusion which was recently verified by x-ray crystallographic analysis (8).

The general acid-base group catalyzing the tautomerization step was postulated to be Lys 258 (for a review see Ref. 6). Crystallographic x-ray data propose either Lys 258 or Tyr 70 (8, 48, see also Fig. 13 in the Discussion section). In contrast to earlier chemical modification studies (39), recent x-ray crystallographic analysis has ruled out histidyl residues as participating directly in the catalytic process (8).

Candidates for substrate binding groups were, in view of the anionic character of the substrates, lysyl (40-42), histidyl (43-45) or arginyl residues (42, 46, 47). Crystallographic analysis using N-(5'-phosphopyridoxyl)-aspartate as an analog of covalent coenzyme substrate intermediates has suggested that the guanidinium groups of Arg 386 and Arg 292 might be the principal binding sites for the proximal and distal carboxylate groups of the substrate, respectively (8, 48). A third guanidinium group (Arg 266) forms an ion pair with the phosphate group of the coenzyme (8). Arg 292 is one of those active site groups which are contributed by the neighboring subunit. Previous chemical modification studies have shown that both the cytosolic (42, 46, 47) and the mitochondrial isoenzyme (46) of AspAT are inactivated by modification of unidentified arginyl residues with various dicarbonyl reagents.

In the present study, Arg 292 was found to be readily modified by phenylglyoxal in the absence of substrates and to be completely protected in the presence of an amino acid/keto acid substrate pair. The catalytic properties of the enzyme with blocked Arg 292 are compatible with the conclusion that this residue is the binding site for the distal carboxylate group of the substrate.

3. Proteolytic Modification of Enzymes

Apart from the key rôle of limited proteolysis in the induction of the biological activities of zymogens, selective proteolytic degradation is an important tool for studying the structure-function relationship of proteins. The prerequisite for selective proteolysis of a folded protein is the presence in an exposed position of a bond corresponding to the specificity of the protease employed. According to Linderstrøm-Lang, there are basically two possibilities how proteolysis can proceed (49):

- One-by-one type: The initial cleavage of a peptide bond destabilizes the protein structure. The residual protein unfolds and is rapidly degraded.
- Zipper mechanism: The initial cleavage is much faster than subsequent degradative reactions. The result is a more or less stable intermediate of high molecular weight.

The second mechanism is characteristic for zymogen activation and for all studies discussed below. The following examples illustrate the type of information which can be obtained by using limited proteolysis as an experimental tool. The best studied case is the digestion of ribonuclease A by subtilisin. Subtilisin cleaves either the peptide bond Ala 20 -

Ser 21 or the bond Ser 21 - Ser 22 in the NH_2 -terminal segment (50-52). The resulting S-peptide occupies a part of the surface of the hydrophobic core of the S-protein (53). The two components as produced by limited proteolysis dissociate in trichloroacetic acid. The separated S-peptide and S-protein are enzymically inactive. On mixing, the two parts recombine to an active enzyme, demonstrating that noncovalent forces maintain the conformation of the protein (50). The relationship between structure and function of carboxypeptidase A was also investigated by limited proteolysis with subtilisin (54). Carboxypeptidase S exhibits changed enzymic characteristics with respect to both esterase and peptidase activity. A conformational change in the protein is suggested to account for these effects brought about by the protease.

Limited proteolysis is also an often used approach for studying the arrangement or interactions of subunits in multienzyme complexes (55-59) or oligomers (e.g. see Refs. 60, 61). In membrane proteins the non-penetrating and exposed part of the enzyme can be identified by its susceptibility to limited proteolysis (62; for a review see also Ref. 63).

Alternatively to an exposed loop or a terminal segment, a stretch of polypeptide chain connecting distinct domains of a protein can be a point of attack by proteases. Such a susceptible linking peptide was found, e.g., in glutamine synthetase (64), glutamate dehydrogenase (65) and leucine aminopeptidase (66).

In the case of mAspAT, recent x-ray crystallographic determination of the spatial structure has shown that in each subunit the NH_2 -terminal segment of the polypeptide chain passes in front of the active site cleft and ends in contact with the neighboring subunit. The contact area of this bridging segment is not contiguous with the main subunit interface. The immediately following stretch of polypeptide chain (residues 14 to 32) runs in exposed position on the surface of the globular

part of the subunit (8; see also Fig. 14 in the Discussion section) suggesting that it might be a favorite target for proteolytic attack in the native state of the enzyme. The presence of two pairs of adjacent lysyl and arginyl residues (68) indicated trypsin to be used as protease. This communication reports the tryptic cleavage of native mAspAT at the expected positions and some of its structural and functional consequences.

EXPERIMENTAL PROCEDURE

1. Enzyme (Preparative and Analytical Procedures)

Mitochondrial AspAT was isolated from chicken heart as described previously (69). Protein concentrations were determined photometrically ($\epsilon_{280, \text{subunit}} = 7.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). The enzymic activity toward aspartate was measured in 50 mM sodium borate or in 50 mM sodium phosphate (pH 7.5) in a coupled assay with malate dehydrogenase (EC 1.1.1.37, obtained from Boehringer), as described previously (11). The pyridoxamine form of the enzyme was prepared by adding 2 mM cysteine sulfinate (70) and subsequent gel filtration on Sephadex G-25, equilibrated with 50 mM sodium borate (pH 7.9) or 50 mM sodium phosphate (pH 7.5). For the preparation of the apoenzyme, the pyridoxamine form was dialyzed twice for 15 h against 0.5 M sodium phosphate (pH 5.1, 4°C). The apoenzyme was reconstituted by adding 1 mM PLP (25°C, 30 min.).

2. Chemicals

Phenylglyoxal hydrate was purchased from Fluka. [^{14}C]Phenylglyoxal hydrate was prepared from [$7\text{-}^{14}\text{C}$]acetophenone (obtained from ICN Pharmaceuticals) by oxidation with selenious acid as described elsewhere (71). After repeated crystallization from toluene, the preparation had a constant specific radioactivity of $5 \times 10^5 \text{ cpm}/\mu\text{mol}$. L-Aspartic acid, oxalacetic acid, pyridoxal-5'-phosphoric acid, and pyridoxamine-5'-phosphoric acid hydrochloride were obtained from Merck; L-glutamic acid, 2-oxoglutaric acid, 4-NN-dimethylaminoazobenzene 4'-isothiocyanate, phenylisothiocyanate, 5,5'-dithiobis-(2-nitrobenzoic acid), 2,5-diphenyloxazole, L-phenylalanine, L-alanine, iodo-

acetic acid, 2,3-butanedione, and 1,2-cyclohexanedione were from Fluka. 2-Methyl-DL-aspartic acid and L-cysteine sulfinic acid were from Sigma, and cyanogen bromide from Pierce. Guanidine hydrochloride was purchased from Schwarz/Mann, α -chymotrypsin from Boehringer, and ion-exchange resin M-72 from Beckman. L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin was obtained from Worthington. The activity of trypsin was assayed by the method of Hummel (72), using p-tosyl-L-arginine methyl ester (Calbiochem) as substrate. The pancreatic trypsin inhibitor (Trasylol) was purchased from Bayer.

3. Modification of Arginyl Residues with 2,3-Butanedione, 1,2-Cyclohexanedione and Phenylglyoxal

Chemical modification was carried out with three different arginine modifying reagents which were added to an enzyme solution of 0.01 to 0.02 mM subunit concentration. The reactions were performed in 50 mM sodium phosphate or in 50 mM sodium borate (pH 7.9, 25°C) by addition of 10 mM 2,3-butanedione or 1.5 mM phenylglyoxal. The reaction of mAspAT with 50 mM 1,2-cyclohexanedione was performed in 50 mM sodium borate (pH 8.5). All modifying reagents were freshly dissolved in the appropriate buffer just before use. The reactions were followed by taking samples at timed intervals and assaying them for enzymic activity, either in phosphate or in borate buffer. The number of modified arginyl residues was determined by amino acid analyses after acid hydrolysis at reduced pressure (6 N HCl, 110°C, 22 h) on a Durrum D-500 analyzer using the standard sodium citrate buffers for protein hydrolysates.

4. Isolation of the Peptide Labeled with [^{14}C]Phenylglyoxal

Two samples of mAspAT were modified with 3 mM [^{14}C]phenylglyoxal during 2.5 h, one in the absence of substrate and the other in the presence of 60 mM aspartate plus 2 mM oxalacetate (0.08 mM subunit concentration in 50 mM sodium borate, pH 7.9, 25°C). The reaction was stopped by removal of excess reagent on a Sephadex G-25 column (2.6 x 60 cm, 10% acetic acid). The labeled enzymes were lyophilized, carboxymethylated (73), and dialyzed against 10% acetic acid. After cleavage with cyanogen bromide (74) the material was lyophilized, dissolved in 70% (v/v) formic acid, and after addition of guanidine hydrochloride to a final concentration of 6 M applied to a Sephadex G-75 column (1.9 x 80 cm, 10% formic acid). The radioactive fractions were pooled, lyophilized, suspended in 50 mM sodium borate (pH 8.5), and digested with chymotrypsin (total 2 x 7%, w/w) at 37°C for 7 h. The reaction was terminated by acidification with concentrated formic acid to a final concentration of 10% (v/v). The peptide mixture was fractionated on a Sephadex G-25 fine column (1.1 x 110 cm, 10% formic acid). The radioactive fractions were lyophilized and dissolved in 0.05 M pyridine acetate, pH 2.5. The solution was applied to a M-72 cation-exchange column (0.9 x 20 cm) equilibrated with 0.05 M pyridine acetate (pH 2.5) at 55°C and developed with a four-chamber gradient, containing 200 ml 0.05 M pyridine acetate (pH 2.5), 200 ml 0.2 M pyridine acetate (pH 3.1), 200 ml 0.5 M pyridine acetate (pH 3.75), and 200 ml 2.0 M pyridine acetate (pH 5.0).

High-voltage electrophoresis was performed on Whatman 3 MM paper at pH 1.9 (acetic acid - formic acid - water, 4:1:45) and at pH 3.5 (pyridine - acetic acid - water, 1:10:89). The fractionated peptides were stained with fluorescamine (Roche) and eluted from the paper with 50% acetic acid.

5. Amino Acid Sequence Determination

The radioactive peptide eluted from paper after the two-dimensional high voltage electrophoresis, was subjected to micro-sequence analysis using the 4-NN-dimethylaminoazobenzene 4'-isothiocyanate/phenylisothiocyanate double coupling method (75). The amino acids were identified by two-dimensional thin-layer chromatography on polyamide sheets from Schleicher and Schüll (76). The solvent for the first-dimension development was water/acetic acid (2:1, v/v) and the solvent for the second-dimension development was toluene/n-hexane/acetic acid (2:1:1, v/v). The 4-NN-dimethylaminoazobenzene 4'-thiohydantoin derivatives of the amino acids were detected as red spots in pmol amounts. For fluorography the chromatographic plates were treated with 7% 2,5-diphenyloxazole (PPO) in acetone; a flashed film was exposed at -80°C (77).

6. Limited Digestion of Aspartate Aminotransferase with Trypsin

Trypsin was added repetitively (0.8 mg in 10 μ l of 0.001 N HCl every 15 min) to an enzyme solution of 8.1 mg in 2 ml 50 mM Tris chloride, pH 8.9, at 37°C. Samples of the reaction mixture were assayed for enzymic activity. After the treatment with trypsin, the mAspAT solution was passed through a Sephadex G-150 column (60 x 2.5 cm) equilibrated with 50 mM sodium phosphate, pH 7.5, 4°C.

7. Automated Edman Degradation

Automatic Edman degradation was performed with a Beckman Sequencer (model 890 B, updated), using the quadrol buffer system and the Beckman peptide program (060275). The chemicals used in the sequence determination were purchased from Beckman

or Fluka. Phenylthiohydantoin amino acid derivatives were identified by high pressure liquid chromatography (Waters Associates, model ALC/GPC-204) according to the procedure of Frank and Strubert (78) and by amino acid analysis on a Durrum D-500 analyzer after acid hydrolysis (6 N HCl containing 0.1% stannous chloride, 150°C, 4 h (79)).

RESULTS

1. Chemical Modification of Arg 292

1.1 Inactivation by Three Different Dicarboxyl Reagents

Treatment of the pyridoxal form of mAspAT with 1.5 mM phenylglyoxal in borate buffer results in inactivation to about 5% of the initial value within 1 h (Fig. 1). Similar inactivation is brought about by other dicarboxyl reagents such as 2,3-butanedione and 1,2-cyclohexanedione (Table II). If the modification with phenylglyoxal or butanedione is performed in phosphate instead of borate the enzymic activity decreases by only ~70% within 1 h. The less efficient inactivation is possibly due to the absence of the stabilizing effect of borate on the adduct between the guanidino group of arginine and the reagent. Such a stabilizing effect has been described previously for butanedione (81) and cyclohexanedione (82). mAspAT modified by any of the three reagents in the presence of borate does not regain activity on removal of excess reagent by gel filtration or dialysis in the presence of 50 mM borate. In the absence of borate, the modification is to some extent reversible (Fig. 2). The enzymic activity of mAspAT which had been reduced to 5% of its initial value by reaction with phenylglyoxal in the presence of borate, was restored to about 30% by gel filtration in 50 mM sodium phosphate (pH 7.9). After modification with butanedione in the presence of borate, 80% of the initial activity was regained on gel filtration with phosphate buffer.

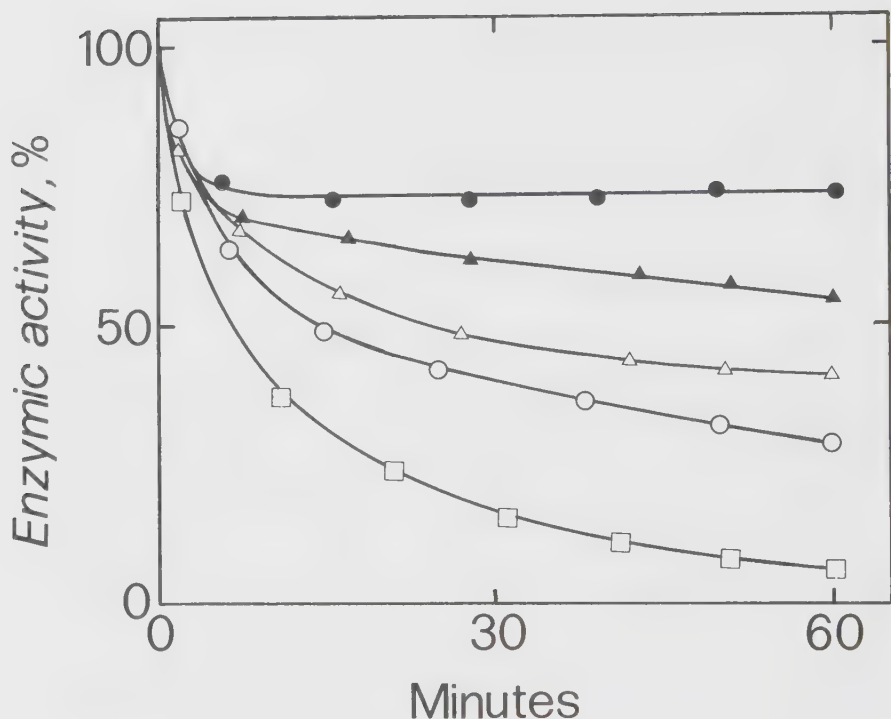


Fig. 1 Chemical modification of mAspAT with phenylglyoxal. The reaction was started by the addition of 1.5 mM phenylglyoxal to a solution of the enzyme (0.02 mM subunit concentration in 50 mM sodium borate, pH 7.9, 25°C) in different functional states: pyridoxal form in the absence of substrate (□), in the presence of 2 mM oxalacetate (○), and in the presence of 30 mM 2-oxoglutarate (Δ); enzyme in the presence of 60 mM aspartate plus 2 mM oxalacetate (●), and enzyme in the presence of 300 mM glutamate plus 30 mM 2-oxoglutarate (▲). The substrate concentrations used are at least 25 times higher than the corresponding K_d values (80).

1.2 Effect of Substrates on the Inactivation by Phenylglyoxal

The rate of inactivation of mAspAT by phenylglyoxal depends markedly on the functional state of the enzyme (Fig. 1). In the presence of oxalacetate or 2-oxoglutarate when nonpro-

TABLE II Inactivation of mAspAT by phenylglyoxal, 1,2-cyclohexanedione or 2,3-butanedione

Conditions of modification	Specific activity after 1 h	Loss of arginyl residues ^a
	% of initial value	mol/mol subunit
<u>Phenylglyoxal, 1.5 mM</u>		
50 mM Sodium borate, pH 7.9	5	2.7
50 mM Sodium phosphate, pH 7.9	30	n.d.
<u>2,3-Butanedione, 10 mM</u>		
50 mM Sodium borate, pH 7.9	0	4.4
50 mM Sodium phosphate, pH 7.9	35	3.7
<u>1,2-Cyclohexanedione, 50 mM</u>		
50 mM Sodium borate, pH 8.5	5	4.6

^aDetermined by amino acid analysis, after removal of excess reagent on a Sephadex G-25 column (10% acetic acid).

ductive enzyme-substrate complexes are formed, the rate of inactivation is decreased. The protective effect is more pronounced in the presence of the transaminating substrate pairs aspartate/oxalacetate or glutamate/2-oxoglutarate. Under all conditions, the decrease in activity is biphasic with an initial fast phase followed by a slower phase. The presence of substrate or of the substrate pair appears to influence mainly the second, slower inhibition process (Fig. 1).

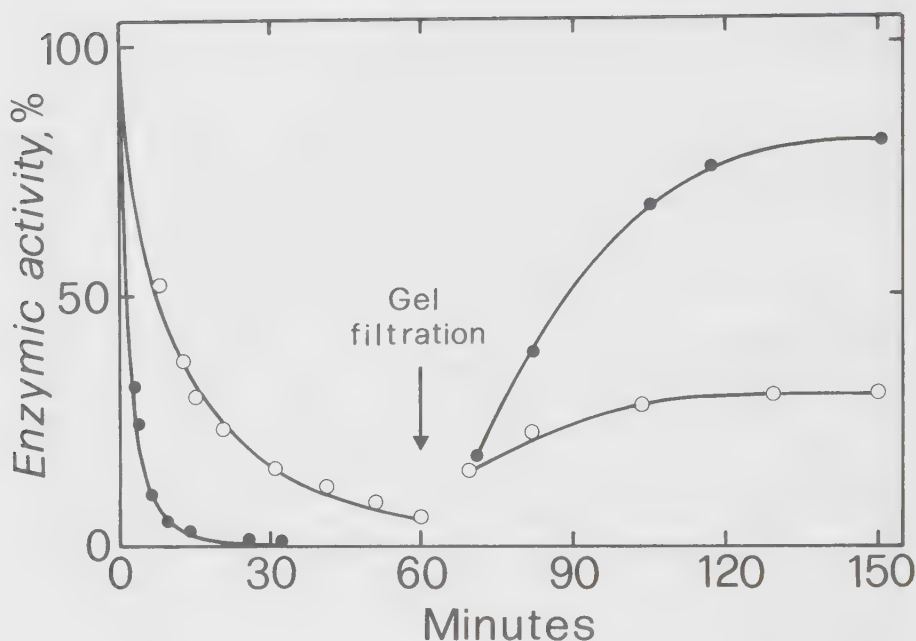


Fig. 2 Reactivation of mAspAT modified with butanedione or phenylglyoxal on removal of borate. The enzyme (0.015 mM subunit concentration) was inactivated with 10 mM 2,3-butanedione (●) or 1.5 mM phenylglyoxal (○) in 50 mM sodium borate, pH 7.9. After 60 min the enzyme solutions were chromatographed on a Sephadex G-25 column (0.6 x 20 cm) equilibrated with 50 mM sodium phosphate, pH 7.9. At the indicated times, samples of the eluted enzymes were taken for measuring the extent of reactivation.

1.3 Modification of the Pyridoxamine Form and the Apoenzyme

The pyridoxamine form of mAspAT is inactivated by phenylglyoxal at about the same rate as the pyridoxal form (Fig. 3). The presence of either aspartate (60 mM) or glutamate (300 mM) retards the inactivation; however, the protective effect is less pronounced than that afforded to the pyridoxal form by oxo acid substrates. In addition to labeling the

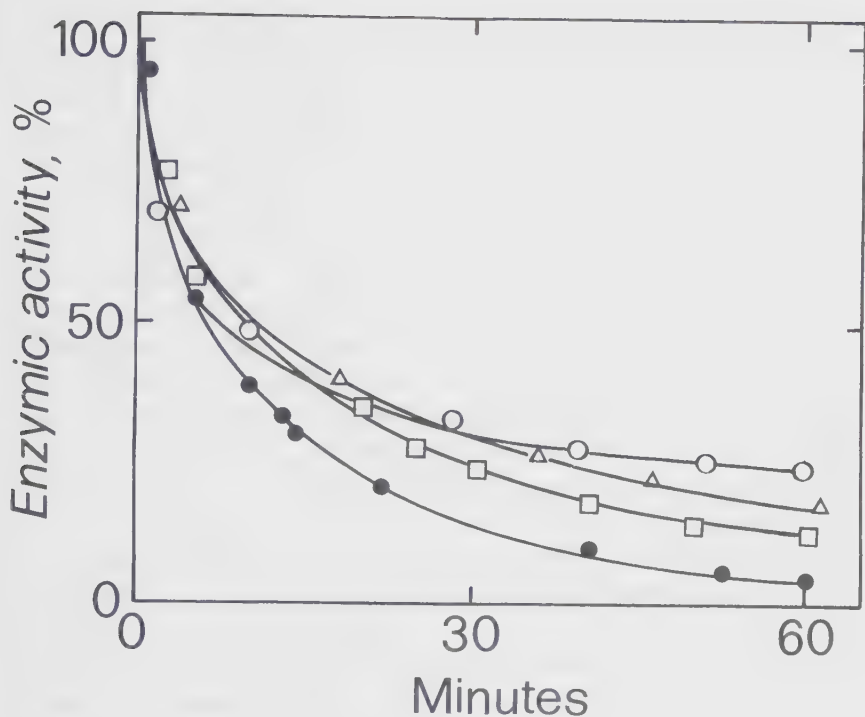


Fig. 3 Modification of the pyridoxamine form and the apoenzyme. The reaction was started by the addition of 1.5 mM phenylglyoxal to the apoenzyme (●) and to the pyridoxamine form in the absence of substrate (□), in the presence of 60 mM aspartate (○), and in the presence of 300 mM glutamate (△).

protein moiety, phenylglyoxal modifies the cofactor pyridoxamine-5'-P. The absorption spectrum of the modified pyridoxamine form showed a maximum at 335 nm with a shoulder at 365 nm and did not change on addition of cysteine sulfinic acid (2 mM). After precipitation of the modified pyridoxamine form with 10% trichloroacetic acid, the resolved coenzyme was analyzed by high voltage paper electrophoresis as described previously (67). The typical orange spot originating from the reaction product of pyridoxamine-5'-P with ninhydrin was

relatively faint; however, a new ninhydrin-negative spot migrating with PLP was found. As is characteristic for PLP, exposure to ammonia vapor produced a yellow color and soaking the paper in sodium borohydride (25 mM in ethanol) produced the intensive bluish fluorescence of the reduction product pyridoxol-5'-P. Phenylglyoxal has been described previously as reacting with amino groups, presumably by undergoing transamination (83). In the present case, phenylglyoxal appears to transaminate with enzyme-bound pyridoxamine-5'-P producing partly an unidentified derivative of the pyridoxal form ($\lambda_{\max}$ 365 nm) and partly a derivative which might be a substituted aldamine ($\lambda_{\max}$ 335 nm, Refs. 67, 84). The latter derivative has been found previously to yield free PLP on denaturation (85).

On treatment with phenylglyoxal, the apoenzyme loses its capacity to be reactivated by PLP at the same rate and to the same extent as the holoenzyme is inactivated (Fig. 3). After removal of excess modifying reagent and addition of PLP (see "Experimental Procedure"), the reconstituted enzyme shows a coenzyme absorption spectrum indistinguishable from that of the native holoenzyme (not shown), indicating that the binding of the coenzyme is not impaired by the modification with phenylglyoxal.

1.4 Quantitation of Modified Arginyl Residues

Amino acid analysis shows that the reaction of dicarbonyl reagents with mAspAT results in a loss of arginyl residues (Table II). The biphasic time-course of the inactivation suggests that more than one residue is modified (Fig. 1). After 1 h of exposure of the unliganded enzyme to phenylglyoxal under the conditions of Fig. 1, 2.7 ± 0.5 arginyl residues (11 analyses from 4 separate experiments) out of a total

of 24 per subunit (24) are lost together with the enzymic activity. If the modification is performed in the presence of aspartate plus oxalacetate, only 1.3 ± 0.6 arginyl residues (9 analyses from 4 experiments) are blocked with 75% of the initial enzyme activity being retained. The amino acid composition of the labeled enzyme gave no indication for modification of amino acid residues other than arginyl. Using [^{14}C]phenylglyoxal, 2.9 mol/mol subunit (3 separate experiments) were found to be incorporated in the absence and 1.3 mol/mol subunit (2 experiments) in the presence of the transaminating substrate pair. Thus, the stoichiometry of the reaction is close to one phenylglyoxal moiety per modified arginyl residue. For free arginine (86) and for some proteins modified in a nonborate buffer (for a review see Refs. 87, 88), two mol phenylglyoxal per mol arginine were reported to be incorporated. The present 1:1 stoichiometry may be due to formation of a complex between the phenylglyoxal arginyl cis-diol adduct and borate, preventing condensation with a second molecule of phenylglyoxal (89). A 1:1 stoichiometry has also been found with a number of other proteins modified in borate or other buffers (for a review see Ref. 90).

1.5 Fragmentation of Labeled Enzyme and Peptide Separation

Two samples of mAspAT were treated with 3 mM [^{14}C]phenylglyoxal during 2.5 h in the absence of substrate and in the presence of 60 mM aspartate plus 2 mM oxalacetate (910 nmol and 1030 nmol mAspAT, respectively). The resulting enzymic activity was 2% and 72% of the initial value, respectively. The prolonged incubation at the higher phenylglyoxal concentration (in comparison with the conditions of Fig. 1), resulted in the incorporation of 3.1 mol [^{14}C]phenylglyoxal per mol subunit in the absence of substrates and of 3.4 mol in their presence. However, amino acid analysis showed that in the latter sample 0.9 mol fewer arginyl residues were modified per mol subunit. The discrepancy might indicate that

one of the arginyl residues modified in the presence of substrates forms an adduct with two phenylglyoxal moieties, or that a modified non-arginyl amino acid residue is deblocked during acid hydrolysis. While the phenylglyoxal arginyl adduct appears to be stable at acidic pH values, arginyl residues are slowly regenerated under neutral or basic conditions (86). In order to reduce the loss of radioactive label during protein fragmentation and peptide isolation, dialysis and gel filtration were carried out under acidic conditions or in borate buffer whenever feasible.

Both the mAspAT sample modified in the absence of the substrate pair and the sample modified in the presence of the substrate pair were subjected to the fragmentation procedure. The yield in radioactivity after carboxymethylation and dialysis (see "Experimental Procedure") was 84% and 55%, respectively, of that measured immediately after removal of excess reagent. The fragments obtained by cyanogen bromide cleavage were fractionated on a Sephadex G-75 column (Fig. 4). The material from the main radioactive peak, containing 62% and 56%, respectively, of the total eluted radioactivity, was pooled and digested with chymotrypsin. The chymotryptic peptides were fractionated on a Sephadex G-25 column (Fig. 5). Chromatography of the sample originating from enzyme labeled in the absence of substrate gave a dominant radioactive peak (47% of the total eluted radioactivity). In the elution profile of the sample modified in the presence of substrates, the radioactivity was spread over a larger range and the corresponding fractions contained only 20% of the total eluted radioactivity. The material of the dominant radioactive peak originating from the protein labeled in the absence of substrate was chromatographed on a M-72 cation exchange column. A single predominant radioactive peak was obtained (Fig. 6). This peak was absent in the corresponding material originating from enzyme modified under substrate protection. The fractions of this peak were pooled and further purified by two-dimensional high voltage paper electrophoresis at pH 1.9

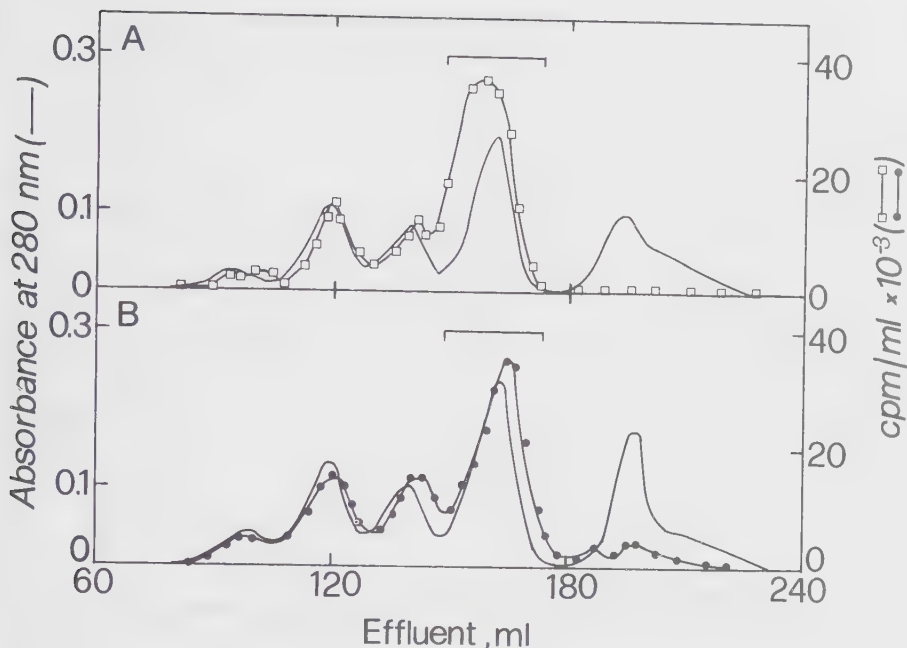


Fig. 4 Chromatography of the cyanogen bromide fragments of mAspAT modified with [^{14}C]phenylglyoxal in the absence of substrate (A), and in the presence of aspartate plus oxalacetate (B) on a Sephadex G-75 column (see "Experimental Procedure"). The fractions that were pooled are indicated by brackets.

and pH 3.5 (see "Experimental Procedure"). On autoradiography, one major spot containing 90% of the total eluted radioactivity and a minor spot were detected. The major spot had the following amino acid composition: proline (1.0), isoleucine (0.65), arginine (0.94), homoserine plus homoserine lactone (0.83). The material was not homogenous due to incomplete chymotryptic cleavage and was composed of a parental tetrapeptide contaminated with a filial tripeptide (see below). It contained 1 mol of [^{14}C]phenylglyoxal per mol of peptide. The phenylglyoxal arginyl adduct seems to be decomposed completely during the acid hydrolysis as indicated by

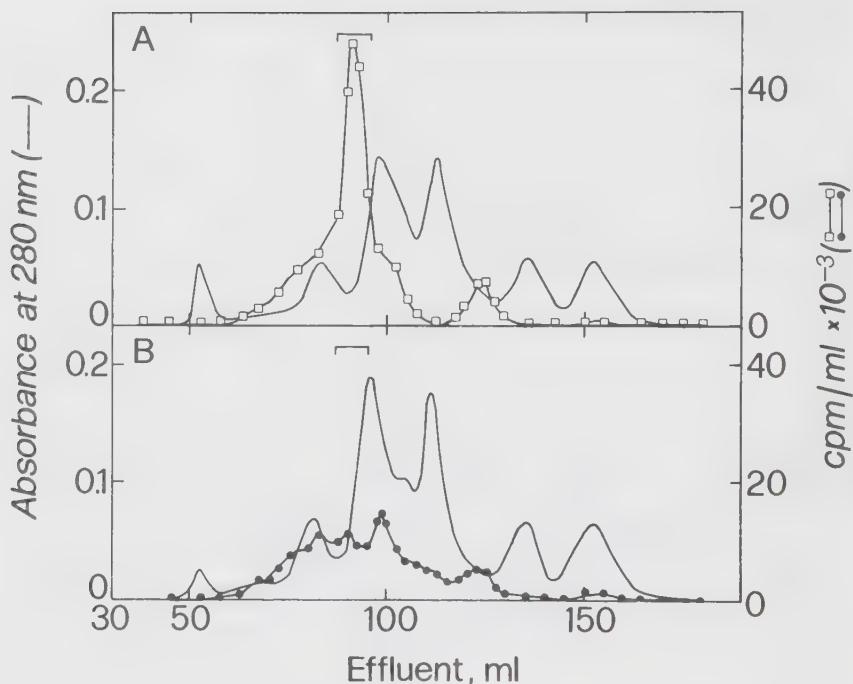


Fig. 5 Chromatography of the chymotryptic peptides from the cyanogen bromide fragments on a Sephadex G-25 fine column (see "Experimental Procedure"). **A**, material from enzyme modified in the absence of substrate; **B**, material from enzyme modified in the presence of aspartate plus oxalacetate. The fractions that were pooled are indicated by brackets.

the occurrence of 1 mol free arginine per mol of proline in the analysis. The lability of the phenylglyoxal arginyl adduct during acid hydrolysis of the isolated peptide contrasts its apparent stability during the acid hydrolysis of the total protein. The basis for the labilization of the adduct during the peptide isolation procedure is unclear. The overall yield of the radioactive peptide after elution from the electrophoresis paper was 44 nmol, i.e., 5% of the starting material, the intact modified enzyme. This low yield is in

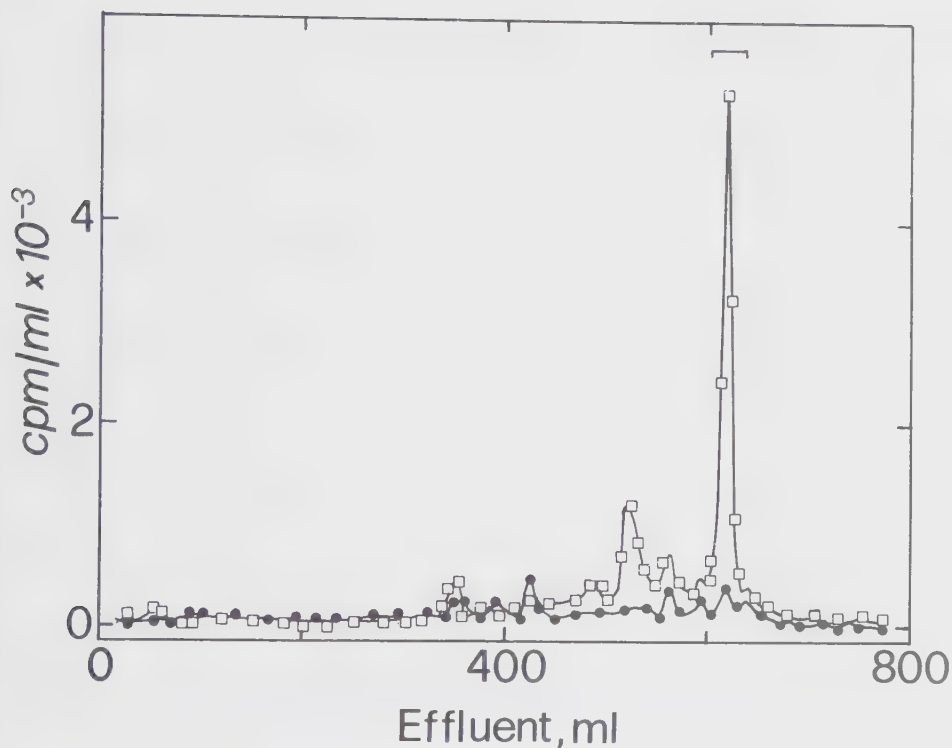


Fig. 6 M-72 cation exchange chromatography of the chymotryptic peptides from the main radioactive pool after purification by gel filtration (see Fig. 5 and "Experimental Procedure"). Peptides from mAspAT modified with [¹⁴C]phenylglyoxal in the absence of substrate, □ ; in the presence of 60 mM aspartate plus 2 mM oxalacetate, ● . The fractions that were pooled are indicated by the bracket.

part due to the succession of a total of four purification steps and in part to the instability of the phenylglyoxal arginyl adduct during the fragmentation and peptide isolation procedure which also has been reported in other cases (86, 91-94).

1.6 Amino Acid Sequence of the Differentially Labeled Peptide

The amino acid sequence of the isolated [^{14}C]phenylglyoxal peptide was determined by three microsequence Edman degradation steps with 4-NN-dimethylaminoazobenzene 4'-isothiocyanate. The amino acid derivatives released in each degradation step were identified by two-dimensional thin-layer chromatography. Their succession indicated the sequence Ile-Arg-Pro-Met. Each degradation step gave two amino acid derivatives originating from the tetrapeptide and the contaminating tripeptide Arg-Pro-Met. After both the first and the second degradation step, a radioactive spot was found on the autoradiographed thin-layer plates which coincided with the colored spot of the derivative of the phenylglyoxal arginyl adduct. The R_f values of this derivative were very similar to those of the derivative of free arginine. The sequence found corresponds uniquely to that of the section Ile₂₉₁-Arg-Pro-Met₂₉₄ in the primary structure of mAspAT from chicken (24). Thus, the residue which is modified in the unliganded pyridoxal form of the enzyme and is completely protected in the presence of the transaminating substrate pair is Arg 292.

The amount of phenylglyoxal incorporated (2.9 mol per subunit in the absence of substrates) indicates that besides Arg 292 other arginyl residues out of a total of 24 (24) are modified by phenylglyoxal. The modification of such additional residues probably accounts for the initial decrease in activity which is not prevented by the presence of the substrates (see Fig. 1).

1.7 Enzymic Activity of Aspartate Aminotransferase with Modified Arg 292

After modification of the pyridoxal form in the absence of substrate, the enzyme is still capable of undergoing a very slow transamination reaction. The shifts of the absorbance

maximum of the enzyme-bound coenzyme from 355 nm (pyridoxal form) to 333 nm (pyridoxamine form) on addition of the amino acid substrate cysteine sulfinic acid and in the reverse reaction on addition of 2-oxoglutarate were followed spectrophotometrically (Fig. 7). The rates of these conversions are 5 orders of magnitude slower than that of the transamination reaction catalyzed by the native enzyme (Table III). Apparently, the residual activity toward aspartate of a few percent of the original value as measured in preparations of the modified enzyme (e.g., see Fig. 1) is due to unlabeled enzyme. In accordance with this conclusion, the apparent Michaelis constants of a preparation of modified enzyme when measured by the coupled assay with aspartate as substrate (see "Experimental Procedure") are the same as those of native mAspAT (0.6 mM for aspartate, at a constant 6 mM concentration of 2-oxoglutarate, and 1.1 mM for 2-oxoglutarate, at a constant 200 mM concentration of aspartate).

The protection of Arg 292 against modification by the transaminating substrate pair indicates that this residue is part of the substrate binding site. In order to examine whether Arg 292 interacts with the distal or with the proximal carboxylate group, the activity of the modified enzyme toward an aromatic and an aliphatic monocarboxylic amino acid was examined (Table III). The relative decrease in activity toward phenylalanine is much smaller than that toward dicarboxylic substrates. The activity toward alanine is not affected by the modification. The decreased activity of the modified enzyme toward the dicarboxylic substrates as well as the low activity of the native enzyme toward alanine is only in part due to weak binding of the substrates rendering it impossible to measure the activity under substrate saturation (Table III).

Formate in high concentration has been reported to enhance the alanine aminotransferase activity of AspAT (95). This increase is thought to be due to interaction of formate with

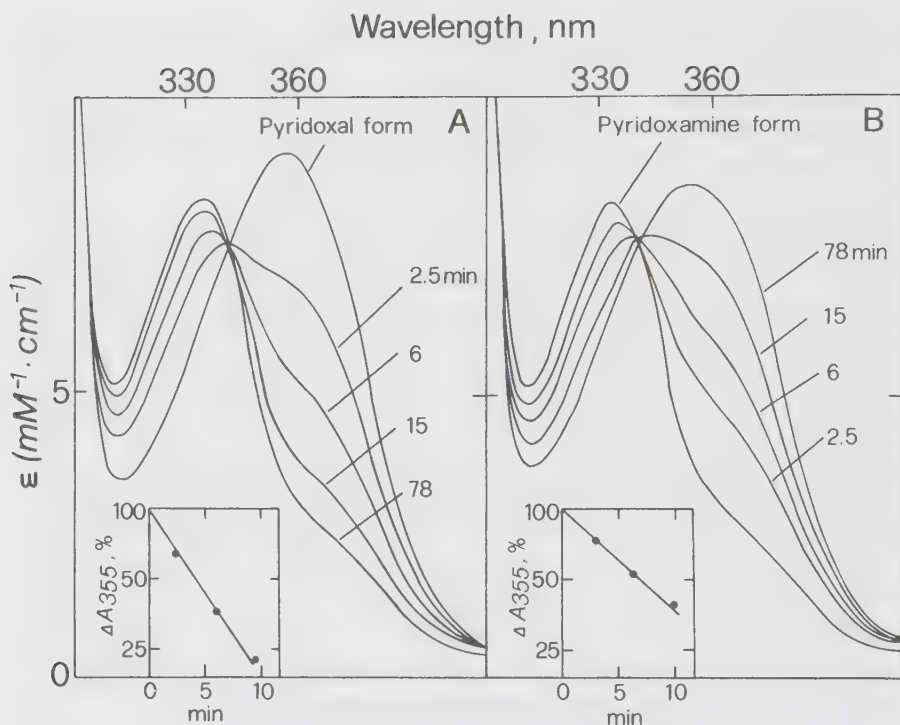


Fig. 7 Half-reactions of transamination undergone by modified mAspAT. After modification in its pyridoxal form (conditions see Fig. 1), the enzyme had a residual activity of 3%. **A:** After recording the spectrum of the pyridoxal form in 50 mM sodium borate, pH 7.9, 25°C, 40 mM cysteine sulfinic acid was added and spectra were recorded at the indicated times which refer to the passing at 355 nm. **B:** The pyridoxamine form thus generated was passed through a Sephadex G-25 column equilibrated with 50 mM sodium borate, pH 7.9. After addition of 40 mM 2-oxoglutarate, the half-reaction back to the pyridoxal form at 25°C was followed spectrophotometrically.

the subsite which normally binds the distal carboxylate group, and to an ensuing conformational change favorable for catalysis (96). In native mAspAT the rate of the half-reaction with alanine is enhanced 7-fold in the presence of 1 M

TABLE III Rate of half-reaction of the modified enzyme
with dicarboxylic and monocarboxylic substrates

The values listed correspond to the ratio between the activity toward the indicated substrates and the molecular activity of native enzyme with 200 mM aspartate and 6 mM 2-oxoglutarate as substrates in 50 mM sodium borate (pH 7.5, 25°C) which is 8900 min^{-1} . The reactions were followed by the change in absorbance at 355 nm of a solution of modified enzyme (0.04 to 0.07 mM) with 3% residual activity in 50 mM sodium borate, pH 7.9, 25°C, after addition of 40 mM cysteine sulfinat, 30 mM phenylalanine, or 65 mM alanine to the pyridoxal form, or of 40 mM 2-oxoglutarate to the pyridoxamine form (see Fig. 7).

Substrate	Relative rate	
	Native enzyme	Modified enzyme
Cysteine sulfinat	n.d.	$1.7 \cdot 10^{-5} \text{ }^a$
2-Oxoglutarate	n.d.	$1.0 \cdot 10^{-5} \text{ }^a$
Phenylalanine	$1.3 \cdot 10^{-4}$	$4.5 \cdot 10^{-6}$
Alanine	$5.6 \cdot 10^{-6}$	$5.6 \cdot 10^{-6}$
Alanine in the presence of 1 M formate	$3.8 \cdot 10^{-5}$	$3.8 \cdot 10^{-5} / 5.6 \cdot 10^{-6} \text{ }^b$

^aHalving the concentrations of cysteine sulfinat and 2-oxoglutarate to 20 mM decreases the rate of the transamination half-reaction by only 30 to 40 percent. The K_m values of the modified enzyme for these substrates are 35mM and 11 mM, respectively.

^bA first fast phase is followed by a second slower phase corresponding to 65% of the total reaction.

formate. Modified mAspAT in the pyridoxal form in the presence of 1 M formate shows a biphasic decrease of the absorbance at 355 nm on addition of alanine (65 mM) reflecting its transformation to the pyridoxamine form. The rate of the initial rapid phase corresponds to an acceleration of the half-reaction as observed with the native enzyme in the presence of formate. In contrast, the rate of the second slower phase (65% of the extent of the total reaction) corresponds to that of the native and the modified enzyme in the absence of formate (see Table III). The partial preservation of the formate effect might reflect that Arg 292 has been transformed into two different derivatives, both of them impairing the activity toward dicarboxylate substrates but only one of them abolishing the rate-enhancing effect of formate. In the enzyme which has been modified under the protection of aspartate and oxalacetate and thus has a free guanidinium group at Arg 292, the rate-enhancing effect of formate is fully operative. A protective effect against inactivation by phenylglyoxal similar to that of the substrate pair aspartate plus oxalacetate is also exerted by 1 M formate or 1 M chloride (Fig. 8). This finding supports the assumption that Arg 292 is a binding site for these anions.

2. Limited Proteolysis of Aspartate Aminotransferase by Trypsin

2.1 Inactivation of Aspartate Aminotransferase by Trypsin

If native mAspAT is treated with trypsin, the enzymic activity decreases progressively. The rate of inactivation depends on the functional state of the enzyme. The pyridoxal form lost 97% of its initial activity within 8 h if exposed to repetitive additions of trypsin (Fig. 9). The progress of inactivation was similar in the case of the pyridoxal form in the

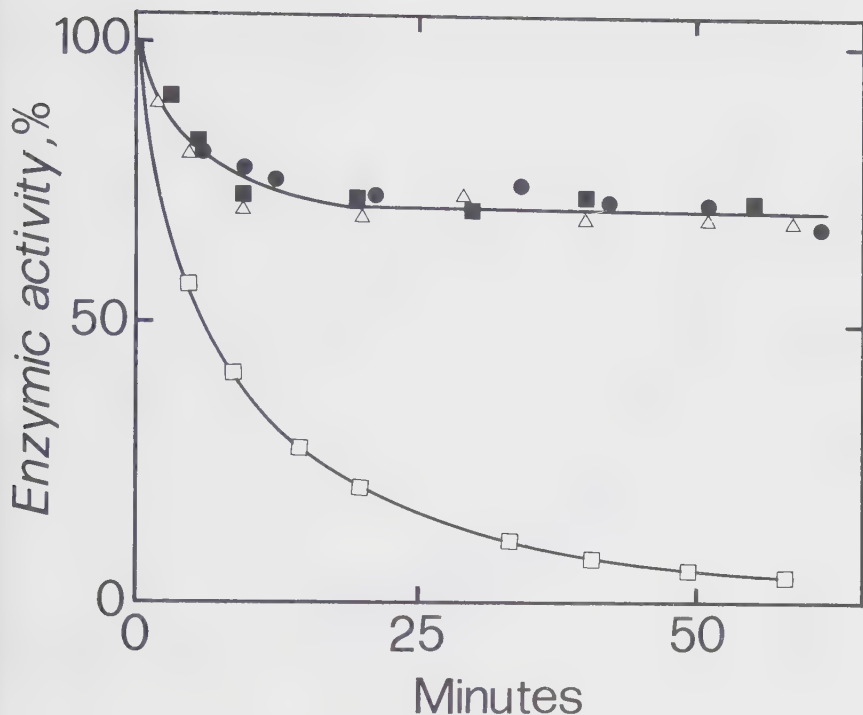


Fig. 8 Effect of anions on the rate of inactivation of mAspAT (pyridoxal form) with phenylglyoxal. The enzyme solution (0.02 mM in 50 mM sodium borate, pH 7.9) was exposed to 1.5 mM phenylglyoxal in the absence of anions (□), in the presence of 1 M formate (△), 1 M chloride (■), and 60 mM aspartate plus 2 mM oxalacetate (●).

presence of oxalacetate and of the pyridoxamine form in the presence or absence of aspartate. However, in the presence of aspartate plus oxalacetate, the inactivation occurred with a marked lag phase and the decrease in activity was about 4 times slower.

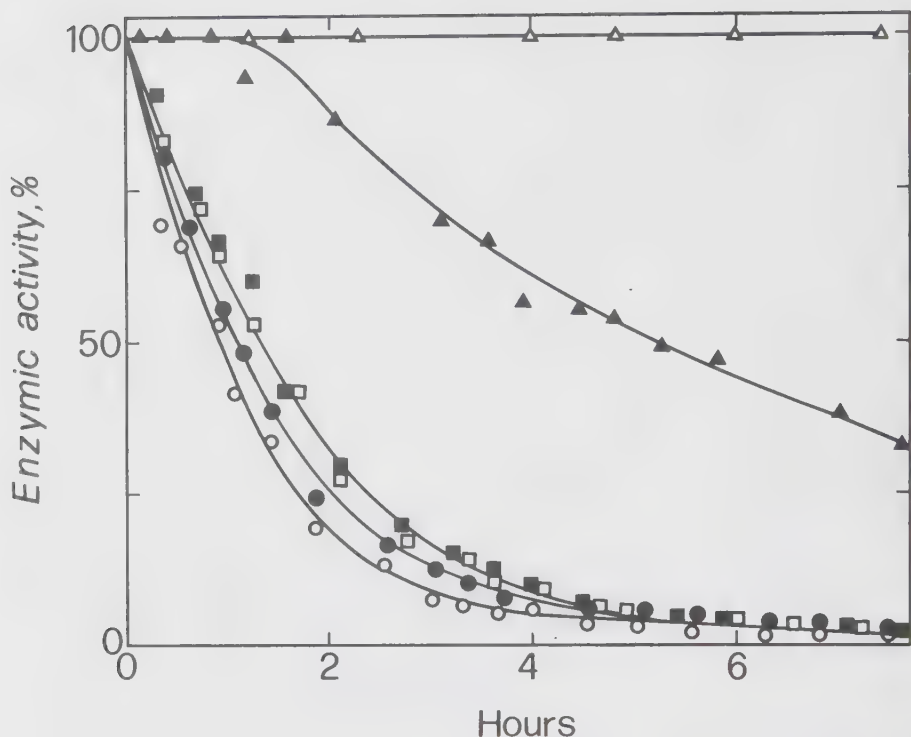


Fig. 9 Digestion of native mAspAT with trypsin. Trypsin was added repetitively to the enzyme (see "Experimental Procedure") in different functional states: pyridoxal form in the absence of substrate ($\square$), and in the presence of 0.4 mM oxalacetate ($\blacksquare$); pyridoxamine form without substrate ($\circ$), and with 11 mM aspartate ($\bullet$); enzyme in the presence of 0.4 mM oxalacetate plus 11 mM aspartate ($\blacktriangle$). The control was kept at 37°C without trypsin and without substrate (Δ). The repetitive addition of relatively large doses of trypsin was necessitated by its rapid autodigestion under the conditions used. At the indicated times a sample of the reaction mixture was assayed for AspAT activity. In the assays the concentration of mAspAT with 3% residual activity was 0.06 μ M (subunit concentration).

2.2 Characterization of the Trypsin-Treated Enzyme

2.2.1 Subunit Molecular Weight

Trypsin cleaves mAspAT at a specific site. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that the loss of enzymic activity correlates with the disappearance of the band of native mAspAT (subunit molecular weight 45,000) and the appearance of a new band with a molecular weight of 42,000 (Fig. 10 A). Temporally, the decrease in enzymic activity immediately follows the peptide bond hydrolysis. The lag phase in inactivation observed in the presence of the transaminating substrate pair (Fig. 9) reflects a retardation in the proteolytic cleavage, as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 10 B). Accordingly, the pancreatic protease inhibitor which blocks trypsin activity in less than 1 min (for assay procedure, see "Experimental Procedure") stopped the inactivation of mAspAT both in the absence and presence of the substrates without noticeable delay (200 μ g Trasylol was added to 2 ml of the mixture of mAspAT and trypsin under the conditions described in Fig. 9). The mechanism by which the substrate pair causes a delay in proteolysis remains unclear on the basis of the present data and is the subject of further investigations.

2.2.2 Identification of the Cleavage Sites

Sequence analysis of trypsin-treated mAspAT by automated Edman degradation revealed the presence of two polypeptide chains, one with aspartate and one with lysine as NH_2 terminus. The amino acid sequences of the following stretch of the two chains (Table IV) can be accommodated uniquely in the primary structure of the enzyme. The new NH_2 -terminal residues, Asp 27 and Lys 32, correspond with cleavage by trypsin after Arg 26 or Lys 31, respectively (Fig. 11).

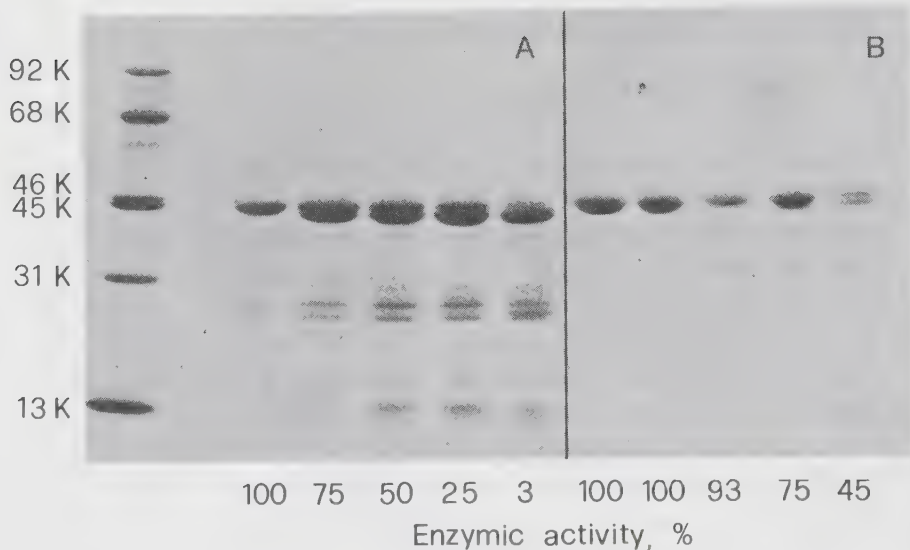


Fig. 10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of mAspAT digested by trypsin. A 7.5%/20% gradient separation gel overlaid with a 3% spacer gel was used. The marker proteins in order of decreasing molecular weight are: glycogen phosphorylase, bovine serum albumin, cytosolic and mitochondrial AspAT, carbonic anhydrase, cytochrome c. The bands with molecular weights below 31,000 are due to trypsin and trypsin degradation products. **A**, During proteolysis of the pyridoxal form of the enzyme in the absence of substrates (Fig. 9), samples were taken from the reaction mixture immediately after the first addition of trypsin and when the enzymic activity had reached 75, 50, 25 and 3 per cent of its initial value. **B**, Samples were taken during proteolysis of the pyridoxal form in the presence of 0.4 mM oxalacetate plus 11 mM aspartate (Fig. 9) at the same times as the samples in **A**. Per slot 12 μ g were loaded except for slot 8 and 10 where only 5 μ g were applied.

No other new NH_2 termini were detected (Table IV). Under the conditions used, AspAT 27 - 410 and AspAT 32 - 410 appear to be produced in about equal amounts. Of course, this finding does not imply that the two bonds are hydrolyzed independently from each other; it is possible that the cleavage of the 31 - 32 bond has to be preceded by the cleavage of the

TABLE IV Automated Edman degradation of trypsin-digested mAspAT after gel filtration

After treatment with trypsin the mAspAT solution (3.5 ml) with 3% residual activity (see Fig. 10 A) was passed through a Sephadex G-150 column (60 x 2.5 cm, 50 mM sodium phosphate, pH 7.5, 4°C). Following dialysis against water a sample of 250 nmol was subjected to automated Edman degradation. Phenylthiohydantoin amino acid derivatives were identified by high pressure liquid chromatography and by amino acid analysis after acid hydrolysis (see "Experimental Procedure"). A total of 25 steps were analyzed with a repetitive yield of 0.97. Neither by high pressure liquid chromatography nor by amino acid analysis after acid hydrolysis were any other residues found to be released except traces of serine and tryptophan originating from undigested protein.

Step number	Amino acid residues released	
1	Asp	Lys
2	Thr	Met (86 nmol) ^a
3	Asn	Asn
4	Ser	Leu (83 nmol) ^a
5	Lys	Gly
6	Lys	Val
7	Met (78 nmol) ^a	Gly
8	Asn	Ala
9	Leu (74 nmol) ^a	Tyr
10	Gly	Arg

^aDetermined from absorbance of the phenylthiohydantoin derivatives at 254 nm after high pressure liquid chromatography using standards of phenylthiohydantoin methionine and leucine. The yield of phenylthiohydantoin amino acids released from both polypeptide chains together is about 70% of the total protein subjected to Edman degradation.

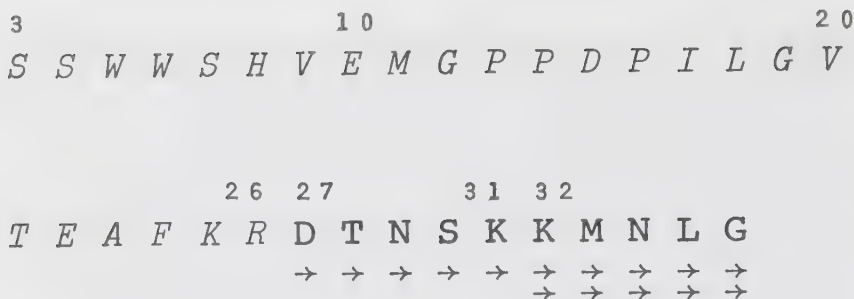


Fig. 11 Comparison of NH₂-terminal sequence of trypsin-digested mAspAT with that of native enzyme. The arrows indicate the two shortened polypeptide chains which were subjected to Edman degradation. For both shortened proteins all 25 steps analyzed (Table IV) corresponded with Asp 27 and Lys 32 being the two new NH₂ termini. For the numbering of the amino acid residues see "Footnote" p 9.

26 - 27 bond. The following experiments were all performed with a mixture of the two shortened enzyme species designated as AspAT 27/32 - 410. After gel filtration under nondenaturing conditions, the cleaved-off peptides (T-peptides) were no longer associated with the rest of the enzyme as shown by the absence of the original NH₂ terminus (Table IV) and the amino acid composition of the shortened enzyme (Table V). The COOH-terminal amino acid sequence of AspAT 27/32 - 410 (Val -Thr - Lys) was found to be identical with that of the native enzyme thus excluding cleavage of peptide bonds close to the COOH terminus (Table VI).

2.3 Enzymic Activity of Aspartate Aminotransferase 27/32 - 410

Spectrophotometric analysis showed that both AspAT 27 - 410 and AspAT 32 - 410 are enzymically competent. On addition of cysteine sulfinic acid (2 mM), the absorption spectrum of the pyridoxal form of the shortened enzyme (20 μM subunit concentration) changed completely into that of the pyridoxamine

TABLE V Deficit in amino acid residues of trypsin-treated mAspAT after gel filtration

Amino acid	Found ^a	Expected ^b
	(mol/mol subunit)	(mol/mol subunit)
Asp	1.9	2
Thr	0.9	1.5
Ser	2.2	3.5
Glu	2.5	2
Pro	1.7	3
Gly	1.6	2
Ala	0.5	1
Val	0.6	2
Met	0.6	1
Ile	0.5	1
Leu	0.7	1
Tyr	0.0	0
Phe	0.6	1
His	1.3	1
Lys	1.4	1.5
Arg	1.2	1
Trp	1.8 ^c	2

^aThe values given represent the differences between native and trypsin-treated enzyme after gel filtration (Table IV). They are average values obtained from seven separate amino acid analyses of two different samples of native and of trypsin-treated enzyme after acid hydrolysis in 6 N HCl in sealed tubes for 22 h at 110°C.

^bCalculated on the basis of the amino acid composition of the native enzyme (see a) and of the amino acid sequence of the NH₂-terminal segment assuming 50% cleavage after Arg 26 and 50% cleavage after Lys 31 (see Fig. 11).

^cTryptophanyl residues were determined spectrophotometrically from the ratio of the coenzyme absorbance at 355 nm to the protein absorbance at 280 nm (0.13 and 0.15 in the native and trypsin-treated enzyme, respectively). The calculation is based on the molar absorptivity of the native enzyme

$$\epsilon_{280, \text{subunit}} = 70 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \text{ and of that of tryptophan}$$

$$\epsilon_{280} = 5.4 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \text{ (97)}.$$

TABLE VI Determination of the COOH-terminal amino acid sequence of native mAspAT and AspAT 27/32 - 410

mAspAT and AspAT 27/32 - 410 (3% residual activity) were passed over a Sephadex G-150 column (60 x 1.5 cm) in 10% (v/v) acetic acid, lyophilized and digested with carboxypeptidase Y (from Boehringer, 1:50, w/w) in 0.1 M 2-(N-morpholino)ethane-sulfonate containing 1% (w/v) sodium dodecyl sulfate (pH 6.3, 22°C). The progress of digestion was followed by amino acid analysis after 5, 15, 45 and 90 min.

	AspAT 27/32 - 410				Native mAspAT			
	5	15	45	90 min	5	15	45	90 min
	Amino acid released (mol/mol protein)							
Lys	0.24	0.44	0.65	0.77	0.26	0.48	0.74	0.89
Thr	0.17	0.41	0.62	0.76	0.16	0.30	0.65	0.81
Val	0.12	0.39	0.59	0.72	0.07	0.17	0.53	0.76

form in less than 20 s; after removal of cysteine sulfinic acid by gel filtration, addition of 2-oxoglutarate (2 mM) resulted in the complete reverse half-reaction back to the pyridoxal form.

The molecular activity of AspAT 27/32 - 410 is concentration-dependent. The activity of a typical preparation (Fig. 9) was 6% of that of the native enzyme if measured at subunit concentrations > 0.3 μ M. However, at lower subunit concentration (0.06 μ M, Fig. 9) it was only 3% of that of the native enzyme. With native enzyme no such concentration dependence of the catalytic activity is found. Apparently, at low concen-

tration, AspAT 27/32 - 410 dissociates into enzymically inactive monomers (unpublished results), the residual activity at low concentration being due to undigested enzyme (see legend of Table IV). Thus, the difference between the activity at high and low enzyme concentration ($6\% - 3\% = 3\%$) represents the activity of AspAT 27/32 - 410 dimers. The dependence of the molecular activity on enzyme concentration was also found when the activity was determined at high and low enzyme concentration as a function of substrate concentration (Fig. 12). The apparent Michaelis constants of an AspAT 27/32 - 410 preparation measured at high subunit concentration ($1.4 \mu\text{M}$, i.e., both AspAT 27/32 - 410 and contaminant undigested enzyme are enzymically active) and at low subunit concentration ($0.03 \mu\text{M}$, i.e., only undigested enzyme is active) are the same as those of the native enzyme, namely 0.8 mM for aspartate (0.2 to 50 mM , at a constant 6 mM concentration of 2-oxoglutarate), and 1.6 mM for 2-oxoglutarate (0.5 to 4.0 mM at a constant 200 mM concentration of aspartate).

The increased dissociation of AspAT 27/32 - 410 into monomers is accompanied by a decrease in heat stability. At 50°C , AspAT 27/32 - 410 (pyridoxal form, 0.8 mM (subunit concentration) in 50 mM sodium phosphate, $\text{pH } 7.5$) loses half of its enzymic activity within 80 min , whereas the native enzyme is not affected.

2.4 Syncatalytic Modification by 5,5'-Dithiobis-(2-nitrobenzoate)

The decreased enzymic activity of AspAT 27/32 - 410 might be due to an impaired capability of the enzyme to undergo the syncatalytic changes in conformation. The reactivity of Cys 166 serving as a probe for syncatalytic conformational adaptations (12) is the same in the enzyme derivative and in the native enzyme (Table VII). However, in AspAT 27/32 - 410 the

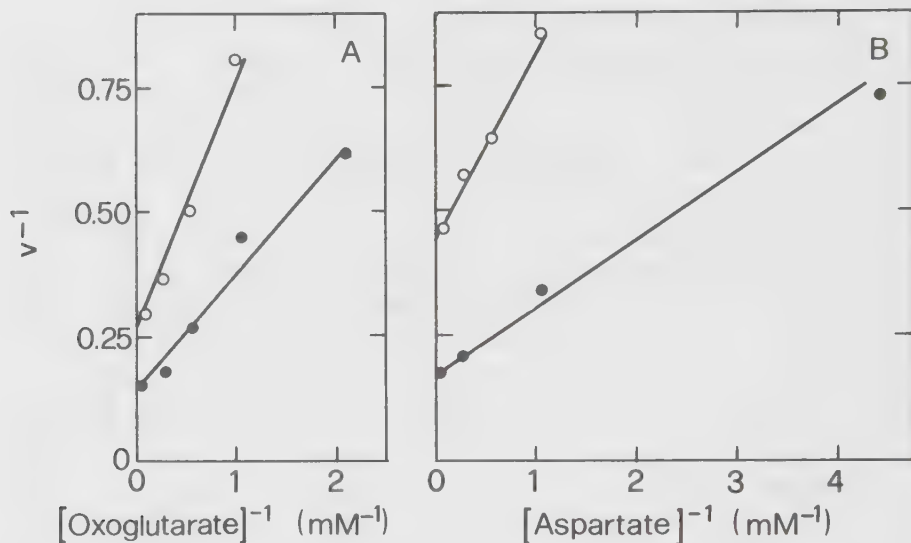


Fig. 12 Dependence of enzymic activity on substrate concentration determined at high and low concentration of AspAT 27/32 - 410. **A**, varying concentrations of 2-oxoglutarate at a constant 200 mM concentration of aspartate; **B**, varying concentrations of aspartate at a constant 6 mM concentration of 2-oxoglutarate. The $V_{\max}$ at low enzyme concentration (0.03 μM subunit concentration, ○) corresponds to a specific activity of 3.0 units/mg in experiment A and 2.3 units/mg in experiment B. The $V_{\max}$ at high enzyme concentration (1.4 μM subunit concentration, ●) corresponds to a specific activity of 6.8 units/mg in experiment A and 5.6 units/mg in experiment B. The apparent Michaelis constants of AspAT 27/32 - 410 for aspartate and for 2-oxoglutarate are 0.8 mM and 1.6 mM, respectively.

reactivity of Cys 166 is markedly desensitized toward the addition of substrates. While in the native enzyme addition of the transaminating substrate pair aspartate and oxalacetate or of the substrate analog 2-methylaspartate which forms an aldimine intermediate (99) results in a 6- to 7-fold increase in reactivity of Cys 166, the corresponding increase in AspAT 27/32 - 410 is only 2-fold.

TABLE VII Syncatalytic conformational changes in native mAspAT and AspAT 27/32 - 410 as probed by the reactivity of Cys 166 toward 5,5'-dithiobis-(2-nitrobenzoate) in different functional states of the enzyme.

The reaction was started by the addition of 5,5'-dithiobis-(2-nitrobenzoate) (1 mM) to an enzyme solution (0.02 to 0.03 mM subunit concentration) containing the indicated substrates in 50 mM sodium phosphate (pH 7.5, 25°C).

Functional state	Relative rate of modification ^a	
	AspAT 27/32 - 410	Native mAspAT
Pyridoxal enzyme	1.1 ^b	1.0 ^b
+ Oxalacetate (0.4 mM)		
and aspartate (11 mM)	2.3	6.1
+ 2-Methylaspartate		
(35 mM)	2.0	6.6
Pyridoxamine enzyme	1.1	1.4

^aThe reaction was followed photometrically (ϵ_{412} of 5-thio-nitrobenzoate = $13,600 \text{ M}^{-1}\text{cm}^{-1}$ (98)). In all cases pseudo-first order kinetics were observed.

^bThe absolute value of the second order rate constant is $45 \text{ M}^{-1} \text{ min}^{-1}$ and $51 \text{ M}^{-1} \text{ min}^{-1}$, respectively. The value for native mAspAT is higher by a factor of 2 than that reported previously (12). The difference might relate to the different preparations of enzyme used (the specific activity of the present preparation is 170 units/mg instead of the earlier 140 units/mg).

DISCUSSION

1. Modification of Arg 292, a Functional Residue at the Active Site

In many proteins, the guanidinium groups of arginyl residues have been found to serve as positively charged loci for the binding of anionic ligands (100, 101). Mitochondrial AspAT is inactivated by three different α -dicarbonyl reagents selectively modifying arginyl residues. For this study, phenylglyoxal was preferred to 1,2-cyclohexanedione or 2,3-butanedione because it inactivated the enzyme with minimum loss of arginyl residues (Table II) and because it can be readily synthesized from a commercially available radioactive precursor. The absence of inactivation when the enzyme is protected by the substrate pair aspartate/oxalacetate correlates with a decrease in the number of arginyl residues modified as indicated by both amino acid analyses ($\Delta = 1.4$ residues per subunit) and measurements of [^{14}C]incorporation ($\Delta = 1.6$ residues per subunit). Arg 292, which is completely protected by the pair of the dicarboxylic substrates, is conserved in all six AspAT sequences that have been determined to date (18-24). The only partial protection of the pyridoxal form by single dicarboxylic keto acid substrates is probably due to their weaker binding compared to the productive enzyme-substrate complexes.

2. Evidence for Arg 292 Being the Binding Site for the Distal Carboxylate Group of the Substrate

The identification of Arg 292 as a substrate binding site is in accord with the x-ray crystallographic studies of the complex of apoenzyme with phosphopyridoxyl aspartate which may be

regarded as an analog of the external aldimine coenzyme-substrate compound. These studies indicated that Arg 292 interacts with the distal carboxylate group of the substrate moiety (Refs. 8 and 48; see also Fig. 13). This residue is contributed by the neighboring subunit. Nevertheless, the two subunits of the AspAT dimer have proven to be functionally independent (102-107).

The experimental evidence obtained by chemical modification which supports the notion of Arg 292 being the binding site for the distal carboxylate group of the substrate may be summarized as follows: (1) The modification of Arg 292 does not affect the interaction of mAspAT with the monocarboxylic substrate alanine. Unimpaired activity toward alanine after modification of arginyl residues has been observed previously with the cytosolic enzyme from chicken (42). (2) The activity of the modified enzyme toward phenylalanine is decreased only 30-fold while the activity toward the dicarboxylic substrates is five orders of magnitude lower than that of the native enzyme. Kinetic studies have indicated that aromatic amino acid substrates bind to a site which does not overlap with the binding site for the distal carboxylate group (108). The binding site for the aromatic ring appears to be provided by the pocket wall opposite to Arg 292 (48) and in the modified enzyme might be partially blocked by the phenylglyoxal moiety linked to Arg 292. (3) Formate which has a rate-enhancing effect on the activity of AspAT toward alanine (95, 96) and aromatic substrates (108) has been suggested to occupy the binding site of the distal carboxylate group of dicarboxylic substrates. The present finding that formate as well as chloride are able to protect the enzyme against inactivation by phenylglyoxal is consonant with this view. Modification of mAspAT under substrate protection does not impair the increase in the activity toward alanine induced by formate. On the other hand, modification of Arg 292 in the absence of substrates abolishes the effect of formate in two thirds of the

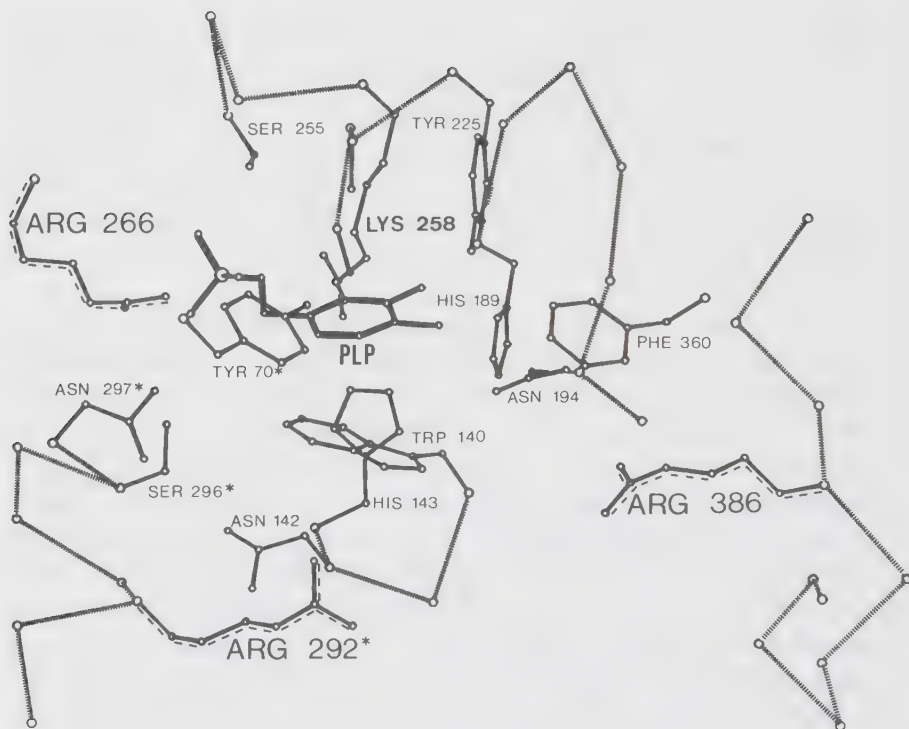


Fig. 13 Amino acid residues at the active site region of mAspAT. The drawing gives a view into the active site cleft. The ring of the coenzyme is seen from above under an angle of $\sim 25^\circ$. Residues belonging to the neighboring sub-unit are indicated by *. The α -carbon backbone is marked with , the strong dark line represents the coenzyme PLP, and the dashed line the three arginyl residues discussed in this study. (Courtesy of Prof. J.N. Jansonius and colleagues, Bio-center, University of Basel).

enzyme molecules (see comments on inhomogeneity of modification under "Results"). Similarly, modification of arginyl residues in the cytosolic enzyme from chicken with 1,2-cyclohexanedione leaves the activity toward alanine intact while partially eliminating the rate-enhancing effect of formate (42).

It should be noted that the modified mAspAT is still capable of catalyzing transamination of the dicarboxylic substrates, though the rate is reduced by 5 orders of magnitude, compared to the native enzyme. The rate of reaction of modified mAspAT toward dicarboxylic substrates is of the same order of magnitude as that of the native enzyme toward alanine. This coincidence is in accord with the previous notion (95) that the interaction of the distal carboxylate group of the substrate with the enzyme is important for the high rate of reaction of the enzyme with its specific, i.e., dicarboxylic, substrates. The rate-enhancing effect of formate on the reaction with alanine (95, 96) is consistent with this view and might indicate that, in addition to a positioning effect on the substrate, the charge interaction might trigger conformational changes essential for efficient catalysis.

Taken together, the present and previous studies on the enzyme in solution and the x-ray crystallographic data (8) indicate that Arg 292 is the binding site for the distal carboxylate group of dicarboxylic substrates. The interaction between the distal carboxylate group and Arg 292 appears to underlie not only the binding specificity but also the kinetic specificity of mAspAT for dicarboxylic substrates.

3. The Rôle of Other Arginyl Residues of Aspartate Aminotransferase

x-Ray crystallographic analysis of the structure of the active site has revealed that in addition to Arg 292 the guanidinium groups of two other arginyl residues are positioned in the proximity of the coenzyme, namely those of Arg 386 and Arg 266 (Ref. 8 and Fig. 13). Difference electron density maps of the phosphopyridoxyl aspartate apoenzyme complex minus phosphopyridoxal holoenzyme indicate that Arg 386 is positioned such as to interact with the α -carboxylate group (8, 48). The fact that the activity toward alanine is not affected by the modi-

fication and the activity toward phenylalanine is less impaired than that toward the dicarboxylic substrates indicates that Arg 386 is still intact. For steric reasons it might not be possible to label Arg 386 if the better accessible Arg 292 (48) is blocked with the bulky phenylglyoxal moiety.

Arg 266 which according to x-ray crystallographic analysis interacts with the phosphate group of the coenzyme (Ref. 8 and Fig. 13) is also not susceptible to phenylglyoxal. Phenylglyoxal-modified apoAspAT retained the capacity to bind pyridoxal phosphate, and the absorption spectrum of the reconstituted modified enzyme was indistinguishable from that of native holoAspAT. Similarly, no arginyl residue interacting with the coenzyme could be modified with phenylglyoxal or 1,2-cyclohexanedione in aspartate aminotransferases from other sources (42, 46, 47).

4. Susceptibility of Aspartate Aminotransferase to Limited Proteolysis

There are two conceivable mechanisms that might render a particular peptide bond in a native protein especially susceptible to proteolytic attack. A sterically exposed position of the bond may favor the approach of the protease or a pronounced flexibility of the polypeptide chain segment may facilitate its accommodation in the active site region of the protease. In mAspAT both mechanisms appear to contribute to the selectivity of its cleavage by trypsin. The enzyme contains a total of 28 lysyl and 24 arginyl residues per subunit (24). The selectively cleaved peptide bonds after Arg 26 and Lys 31 are both exposed to the solvent (Fig. 14). Arg 26 is the 3rd last residue of a short α -helix and Lys 31 is situated in a segment of extended chain (8). The loss of the split-off NH_2 -terminal peptides (T-peptides) from the rest of the protein during gel filtration might indicate that in the intact protein the NH_2 -terminal segment intermittently dissociates

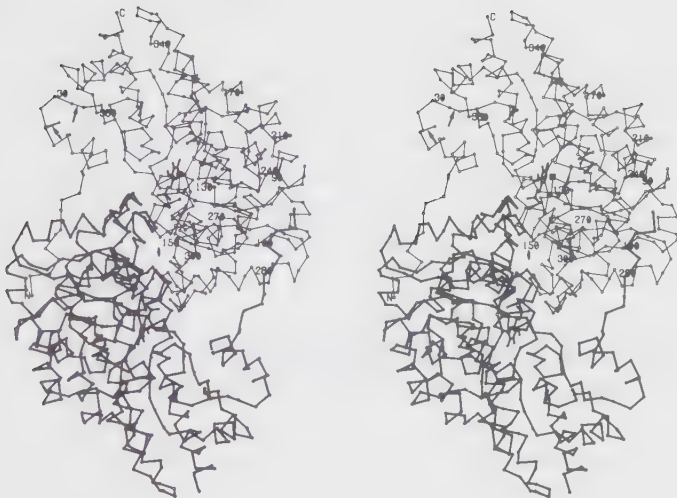


Fig. 14 The spatial structure of mAspAT: the location of the peptide bonds cleaved by trypsin. The stereo drawing of the α -carbon skeleton (courtesy of Drs G.C. Ford, G. Eichele and J.N. Jansonius, Biocenter, University of Basel) is based on a 3.2 Å electron density map of the pyridoxal form of the enzyme. The enzyme dimer is viewed along the molecular diad (). The backbones of the two subunits are indicated by single and by double lines. The other designations are: N, NH₂ terminus; C, COOH terminus; ■, Lys 258 at the active site, the residue binding PLP covalently (67); ★, Cys 166; the arrows indicate the sites of cleavage by trypsin after Arg 26 and Lys 31.

from its inter- and intra-subunit contacts and thus increases in conformational flexibility. The above-mentioned explanations of the observed specificity of the proteolytic cleavage imply that it might not be restricted to trypsin because the discussed factors may be expected to be operative also with other proteases.

The release of the T-peptide distinguishes the case of AspAT 27/32 - 410 from that of the digestion of ribonuclease A with

subtilisin. In ribonuclease A, the subtilisin-susceptible peptide bonds Ala 20 - Ser 21 (50) and to a lesser extent Ser 21 - Ser 22 (51, 52) are also situated in an exposed loop of the polypeptide chain (53, 109). However, in contrast to mAspAT the NH₂-terminal segment is more tightly bound to the rest of the protein and apparently cannot unfold (110). Accordingly, the S-peptide remains bound to the S-protein after cleavage (111).

5. Hypothetical Functional and Structural Rôle of the NH₂-Terminal Segment of the Polypeptide Chain

The NH₂-terminal segment of mAspAT seems to be of both functional and structural significance. The molecular activity of the AspAT 27/32 - 410 dimer is only 3% of that of the native enzyme although none of the amino acid residues in the NH₂-terminal segment appears to be involved directly in the active site (Fig. 14). The K'_m values for aspartate and 2-oxoglutarate are unchanged suggesting an undistorted geometry of the substrate binding site. The decrease in enzymic activity might reflect an interference with the conformational dynamics of the enzyme due to the loss of the bridging peptide. The following experimental evidence points to a rôle of the bridging peptide in the syncatalytic conformational adaptations of mAspAT: (1) The formation of covalent transamination intermediates in the presence of aspartate and oxalacetate retards the rate of the proteolytic cleavage about 4-fold (Fig. 9). This effect is absent when only (nonproductive) enzyme substrate adsorption complexes are formed. After Cys 166 (12) and Cys 390 (present only in cytosolic AspAT from pig, Ref. 11), the NH₂-terminal bridging segment is thus the third surface region of the AspAT molecule which has proven to be involved in the syncatalytic changes in conformation. (2) The difference electron density map of the phosphopyridoxyl aspartate derivative of the enzyme (complex of apoenzyme with phosphopyridoxyl aspartate as an analog of covalent transamination

intermediates) shows in comparison to the pyridoxal form significant conformational changes involving, among other regions, the NH_2 -terminal segment (48, 112). (3) Cys 166, a residue which is situated 25 Å away from the active site (Fig. 14, Ref. 113) and serves as an indicator for syncatalytic conformational transitions (12) shows less pronounced syncatalytic reactivity changes in AspAT 27/32 - 410 (Table VII). Conceivable rôles of the bridging segment in catalysis are those of a mediator between the structural rearrangements at the active site accompanying the covalency changes (9) and more distant parts of the enzyme protein or of a syncatalytically adjustable barrier at the entrance to the active site pocket.

The two additional intersubunit contacts established by the bridging peptides of the two subunits contribute to the stability of the quaternary structure of mAspAT. The decrease in molecular activity at lower concentrations of AspAT 27/32 - 410 apparently reflects the dissociation of the catalytically active enzyme dimer into inactive monomers. In the same concentration range the molecular activity of the native enzyme is not affected by dilution (unpublished results). The conclusion that monomeric mAspAT is catalytically inactive remains to be confirmed; it is, however, supported by other lines of evidence. The x-ray crystallographic elucidation of the spatial structure of mAspAT has shown that the active site is situated in a pocket adjacent to the subunit interface. The major part of one wall of the pocket is made up by residues of the neighboring subunit which contributes part of the potentially functional residues (8). Furthermore, matrix-linked subunits of AspAT have recently been reported to be catalytically inactive (114). Facilitated dissociation of the AspAT 27/32 - 410 dimer might also underlie its lowered resistance to heat denaturation.

Another possible rôle of the NH_2 -terminal segment is indicated by an interspecies comparison of the mitochondrial and the cytosolic isoenzymes of AspAT. The two isoenzymes are homologous

proteins (18-24). In mitochondrial aspartate aminotransferases the amino acid sequence of the NH_2 -terminal segment is highly invariant. In the mitochondrial enzymes from pig and chicken the first 29 residues corresponding to the longer T-peptide differ in only one position (22, 24); whereas, in the same stretch of polypeptide chain the cytosolic enzymes differ in 11 residues (18-20). This high degree of sequence similarity in mitochondrial aspartate aminotransferases perhaps reflects some function of the NH_2 -terminal segment in the selective uptake from its site of synthesis in the cytosol into the mitochondrial matrix (15, 16).

The x-ray crystallographic analysis of cytosolic AspAT both from chicken and pig is currently underway (26, 27); a comparison of the NH_2 -terminal region of the mitochondrial and cytosolic isoenzymes will thus be possible in the near future. The cytosolic isoenzymes from chicken and pig contain trypsin-susceptible peptide bonds in similar positions as the mitochondrial isoenzyme (18-20). Exploratory experiments have shown that limited proteolytic cleavage similar to that observed in the present case also occurs with cytosolic aspartate aminotransferases (L. Meer and H. Gehring, personal communication). AspAT 27/32 - 410, an enzyme derivative which catalyzes transamination at an overall rate 30 times slower than the native enzyme, might prove a valuable object of study for the characterization of the elementary steps of transamination.

SUMMARY

Mitochondrial aspartate aminotransferase as a prototype of a pyridoxal-5'-phosphate (vitamin B₆)-dependent enzyme was examined by both selective chemical modification of an amino acid residue at the active site and proteolytic modification of a part of the enzyme not being involved directly in the active site. The goal of my work was to contribute to an understanding of the rôle of the apoenzyme moiety in the catalysis of transamination.

Mitochondrial aspartate aminotransferase is inactivated by dicarbonyl reagents selectively modifying arginyl residues. Treatment with phenylglyoxal decreases the catalytic activity to 5% of the initial value. This residual activity is mostly due to unmodified enzyme. The inactivation is accompanied by modification of 2.7 mol arginyl residues per mol subunit. If the reaction is performed in the presence of the transaminating substrate pair aspartate/oxalacetate only 1.3 mol arginyl residues per mol subunit are labeled and the enzymic activity remains at 75% of the original value. One particular residue, identified by peptide analysis as Arg 292, is completely protected against modification in the presence of the substrate pair indicating a rôle of its guanidinium group in substrate binding. Arg 292 is known from x-ray crystallographic studies at 2.8 Å resolution to lie in the active site cleft. On the basis of the difference electron density map of the complex of apoenzyme with N-(5'-phosphopyridoxyl)-aspartate (minus pyridoxal form of the enzyme), this residue has been proposed as a binding site of the distal carboxylate group (Ford, G.C., Eichele, G., and Jansonius, J.N. (1980) *Proc. Natl. Acad. Sci.* 77, 2559-2563). The enzyme with blocked Arg 292 is not completely inactive; its very low molecular activity toward dicarboxylic substrates is of the same order of magnitude as that of the native enzyme toward alanine which is 10⁵ times lower than that toward dicarboxylic substrates. The modification does not

affect the activity toward alanine but impairs the rate-enhancing effect of formate on the transamination of alanine. Formate is assumed to occupy the binding site of the distal carboxylate group (Morino, Y., Osman, A.M., and Okamoto, M. (1974) *J. Biol. Chem.* 249, 6684-6692). Apparently, the interaction of the distal carboxylate group of the substrate with Arg 292 underlies not only the binding specificity but also the kinetic specificity of aspartate aminotransferase for dicarboxylic substrates.

Native mitochondrial aspartate aminotransferase is selectively cleaved by trypsin at the peptide bonds after Arg 26 or after Lys 31 yielding two shortened enzyme derivatives, aspartate aminotransferase 27 - 410 and aspartate aminotransferase 32 - 410. Recent x-ray crystallographic determination of the spatial structure of aspartate aminotransferase has shown that the NH₂-terminal segments of the two polypeptide chains of this dimeric enzyme pass in front of the active site clefts and form two separate junctions with the neighboring subunit which are not contiguous with the main subunit interface (Ford, G.C., Eichele, G., Jansonius, J.N. (1980) *Proc. Natl. Acad. Sci.* 77, 2559-2563). The peptide bonds cleaved by trypsin are situated in the following stretch of the polypeptide chain which runs in exposed position on the surface of the subunit. The split-off peptide is lost during gel filtration.

The molecular activity of aspartate aminotransferase 27/32 - 410 (a mixture of about equal amounts of the two not readily separable derivatives) is about 3% of that of the native enzyme. In contrast, the K_m' values for aspartate and 2-oxoglutarate are unchanged, indicating an unaltered geometry of the substrate binding site. A substantially diminished syncatalytic response of the reactivity of Cys 166 toward 5,5'-dithiobis-(2-nitrobenzoate) suggests that the decrease in catalytic activity is due to an interference with the syncatalytic conformational dynamics observed previously in aspartate aminotransferase (Gehring, H., and Christen, P. (1978) *J. Biol. Chem.* 253, 3158-3163). Consonant with a rôle of the NH₂-terminal segment in propagating the syncatalytic

conformational rearrangements, the rate of the tryptic cleavage is retarded 4-fold in the presence of the transaminating substrate pair aspartate and oxalacetate.

ZUSAMMENFASSUNG

Mitochondriale Aspartat-Aminotransferase wurde als Prototyp eines Pyridoxal-5'-phosphat (Vitamin B₆)-abhängigen Enzyms mittels chemischer und proteolytischer Modifikationen untersucht. Während die selektive chemische Modifikation eine Aminosäure an der aktiven Stelle betraf, wurde durch die proteolytische Modifikation ein Enzymteil angegangen, der nicht direkt zur aktiven Stelle gehört. Das Ziel meiner Arbeit war, einen Beitrag zum Verständnis der Rolle des Apoenzymteils bei der Katalyse der Transaminierung zu erbringen.

Dicarbonylreagenzien, die selektiv Argininreste modifizieren, inaktivieren die mitochondriale Aspartat-Aminotransferase. Eine Behandlung mit Phenylglyoxal vermindert die katalytische Aktivität auf 5% des Ausgangswerts. Diese Restaktivität ist grösstenteils auf nicht modifiziertes Enzym zurückzuführen. Die Inaktivierung ist mit der Modifikation von 2.7 mol Argininresten/mol Untereinheit verbunden. In Gegenwart des transaminierenden Substratpaares Aspartat/Oxalacetat reagieren nur 1.3 mol Argininreste/mol Untereinheit, und die Enzymaktivität bleibt auf 75% des Ausgangswerts. Ein einziger Rest, der mittels Peptidanalyse als Arg 292 identifiziert worden ist, ist in Anwesenheit des Substratpaares vollständig geschützt gegen die Modifikation. Aus diesem Befund lässt sich schliessen, dass die Guanidiniumgruppe dieses Rests an der Bindung des Substrats beteiligt ist. Die röntgenkristallanalytische Strukturermittlung bei 2.8 Å Auflösung hat ergeben, dass Arg 292 in der Spalte der aktiven Stelle liegt. Aufgrund der Differenz-Elektronendichtekarte des Komplexes zwischen Apoenzym und N-(5'-Phosphopyridoxyl)-Aspartat (minus die Pyridoxalform des Enzyms) ist dieser Rest als Bindungsstelle für die distale Carboxylatgruppe des Substrats vorgeschlagen worden (Ford, G.C., Eichele, G., und Jansonius, J.N. (1980) Proc. Natl. Sci. 77, 2559-2563). Das Enzym mit blockiertem Arg 292 ist nicht vollständig inaktiv, seine sehr geringe molekulare Aktivität gegenüber Dicar-

bonsäuresubstraten ist von der gleichen Grössenordnung wie diejenige des nativen Enzyms gegenüber Alanin. Die Aktivität des nativen Enzyms gegenüber Alanin ist 10^5 mal geringer als diejenige gegenüber Dicarbonsäuresubstraten. Die Modifikation beeinflusst die Aktivität gegenüber Alanin nicht, beeinträchtigt jedoch den geschwindigkeits-beschleunigenden Effekt von Formiat auf die Transaminierung von Alanin. Von Formiat nimmt man an, dass es die Bindungsstelle für die distale Carboxylatgruppe besetzt (Morino, Y., Osman, A.M., und Okamoto, M. (1974) J. Biol. Chem. 249, 6684-6692). Es scheint, dass die Wechselwirkung zwischen distaler Carboxylatgruppe des Substrats und Arg 292 nicht nur der Bindungsspezifität der Aspartat-Aminotransferase, sondern auch der kinetischen Spezifität des Enzyms für Dicarbonsäuresubstrate zugrunde liegt.

Trypsin spaltet in der mitochondrialen Aspartat-Aminotransferase selektiv die Peptidbindungen nach Arg 26 oder Lys 31. Daraus ergeben sich zwei verkürzte Enzymderivate, Aspartat-Aminotransferase 27 - 410 und Aspartat-Aminotransferase 32 - 410. Gemäss dem durch Röntgenkristallanalyse ermittelten Modell der räumlichen Struktur der Aspartat-Aminotransferase verlaufen die NH_2 -terminalen Segmente der zwei Polypeptidketten des dimeren Enzymmoleküls über die Spalten, in denen die aktiven Stellen liegen. Mit der Nachbaruntereinheit bilden sie zwei separate Kontakte, die nicht mit der Hauptkontaktfläche zwischen den beiden Untereinheiten zusammenhängen (Ford, G.C., Eichele, G., und Jansonius, J.N. (1980) Proc. Natl. Acad. Sci. 77, 2559-2563). Die durch Trypsin gespaltenen Peptidbindungen liegen im nachfolgenden Abschnitt der Polypeptidkette, der in exponierter Lage über die Oberfläche der Untereinheit verläuft. Das abgespaltene Peptid geht während einer Gelfiltration verloren.

Die molekulare Aktivität der Aspartat-Aminotransferase 27/32 - 410 (ein Gemisch ungefähr gleicher Teile von zwei nicht einfach voneinander zu trennenden Derivaten) ist 3% der Aktivität des nativen Enzyms. Die gleichbleibenden K_m^1 -Werte für Aspartat und 2-Oxoglutarat weisen jedoch darauf hin, dass die Geometrie der

aktiven Stelle unverändert bleibt. Eine wesentliche Herabsetzung der synkatalytischen Reaktivität von Cys 166 gegenüber 5,5'-Dithiobis-(2-nitrobenzoat) deutet darauf hin, dass die verminderte katalytische Aktivität auf eine Beeinträchtigung der synkatalytischen Konformationsänderungen des Enzyms (Gehring, H., und Christen, P. (1978) J. Biol. Chem. 253, 3158-3163) zurückzuführen ist. In Uebereinstimmung mit der Funktion des NH₂-terminalen Segments als Vermittler von konformationellen Anpassungen des Enzyms während der Katalyse ist die Geschwindigkeit der tryptischen Spaltung in Gegenwart des Substratpaars Aspartat/Oxalacetat um einen Faktor 4 herabgesetzt.

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